Julius-Maximilians-Universität Würzburg

Characterization of virulence-associated traits of *Escherichia coli* bovine mastitis isolates

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submitted by

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Julius-Maximilians-Universität Würzburg

Charakterisierung virulenz-assozierter Eigenschaften von *Escherichia coli* Isolaten boviner Mastitis

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades
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I hereby confirm that my thesis entitled *´Characterization of virulence-associated traits of Escherichia coli bovine mastitis isolates´* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.
Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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Hiermit erkläre ich an Eides statt, die Dissertation *´Characterization of virulence-associated traits of Escherichia coli bovine mastitis isolates´* eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.
Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

No matter how many mistakes you make or how slowly you progress,

you are still way ahead of everyone who is not trying.

-Anthony Robbins

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I. SUMMARY

Bacterial mastitis is caused by invasion of the udder, bacterial multiplication and induction of inflammatory responses in the bovine mammary gland. Disease severity and the cause of disease are influenced by environmental factors, the cow's immune response as well as bacterial traits. *Escherichia coli* (*E. coli*) is one of the main causes of acute bovine mastitis, but although pathogenic *E. coli* strains can be classified into different pathotypes, *E. coli* causing mastitis cannot unambiguously be distinguished from commensal *E. coli* nor has a common set of virulence factors been described for mastitis isolates. This project focussed on the characterization of virulence-associated traits of *E. coli* mastitis isolates in comprehensive analyses under conditions either mimicking initial pathogenesis or conditions that *E. coli* mastitis isolates should encounter while entering the udder.

Virulence-associated traits as well as fitness traits of selected bovine mastitis or faecal *E. coli* strains were identified and analyzed in comparative phenotypic assays. Raw milk whey was introduced to test bacterial fitness in native mammary secretion known to confer antimicrobial effects. Accordingly, *E. coli* isolates from bovine faeces represented a heterogeneous group of which some isolates showed reduced ability to survive in milk whey whereas others phenotypically resembled mastitis isolates that represented a homogeneous group in that they showed similar survival and growth characteristics in milk whey. In contrast, mastitis isolates did not exhibit such a uniform phenotype when challenged with iron shortage, lactose as sole carbon source and lingual antimicrobial peptide (LAP) as a main defensin of milk. Reduced bacterial fitness could be related to LAP suggesting that bacterial adaptation to an intramammary lifestyle requires resistance to host defensins present in mammary secretions, at least LAP.

E. coli strain 1303 and ECC-1470 lack particular virulence genes associated to mastitis isolates. To find out whether differences in gene expression may contribute to the ability of *E. coli* variants to cause mastitis, the transcriptome of *E. coli* model mastitis isolates 1303 and ECC-1470 were analyzed to identify candidate genes involved in bacterium-host interaction, fitness or even pathogenicity during bovine mastitis.

DNA microarray analysis was employed to assess the transcriptional response of *E. coli* 1303 and ECC-1470 upon cocultivation with MAC-T immortalized bovine mammary gland epithelial cells to identify candidate genes involved in bacterium-host interaction. Additionally, the cell adhesion and invasion ability of *E. coli* strain 1303 and ECC-1470 was investigated. The transcriptonal response to the presence of host cells rather suggested competition for nutrients and oxygen between *E. coli* and MAC-T cells than marked signs of adhesion and invasion. Accordingly, mostly fitness traits that may also contribute to efficient colonization of the *E. coli* primary habitat, the gut, have been utilized by the mastitis isolates under these conditions.

In this study, RNA-Seq was employed to assess the bacterial transcriptional response to milk whey. According to our transcriptome data, the lack of positively deregulated and also of true virulence-associated determinants in both of the mastitis isolates indicated that *E. coli* might have adapted by other means to the udder (or at least mammary secretion) as an inflammatory site. We identified traits that promote bacterial growth and survival in milk whey. The ability to utilize citrate promotes fitness and survival of *E. coli* that are thriving in mammary secretions. According to our results, lactoferrin has only weak impact on *E. coli* in mammary secretions. At the same time bacterial determinants involved in iron assimilation were negatively regulated, suggesting that, at least during the first hours, iron assimilation is not a challenge to *E. coli* colonizing the mammary gland. It has been hypothesized that cellular iron stores cause temporary independency to extracellular accessible iron. According to our transcriptome data, this hypothesis was supported and places iron uptake systems beyond the speculative importance that has been suggested before, at least during early phases of infection. It has also been shown that the ability to resist extracytoplasmic stress, by oxidative conditions as well as host defensins, is of substantial importance for bacterial survival in mammary secretions.

In summary, the presented thesis addresses important aspects of host-pathogen interaction and bacterial conversion to hostile conditions during colonization of the mastitis inflammatory site, the mammary gland.

I.b. ZUSAMMENFASSUNG

Bei der bakteriellen Mastitis handelt es sich um eine Infektion der bovinen Milchdrüse, ausgelöst durch Eintritt und Wachstum der Bakterien im Euter der Kuh. Krankheitsverlauf und Ursache werden beeinflusst durch Umweltfaktoren, das Immunsystem des Wirtes und die Eigenschaften des bakteriellen Erregers. Die Spezies *Escherichia coli* (*E. coli*) ist einer der häufigsten Erreger der akuten bovinen Mastitis. Generell können pathogene *E. coli* -Stämme entsprechend ihres Infektionsortes in verschiedene Pathotypen klassifiziert werden, die durch eine individuelle Kombination verschiedener Virulenzfaktoren gekennzeichnet sind. Eine eindeutige Unterscheidung von *E. coli* –Mastitiserregern und kommensalen *E. coli* -Stämmen ist bisher nicht beschrieben. Diese Studie befasst sich mit der Charakterisierung virulenz-assozierter Eigenschaften von *E. coli* –Isolaten der bovinen Mastitis. Dazu wurden Untersuchungen unter Bedingungen durchgeführt, die denen während der Anfangsphase der Mastitis entsprechen.

Die Virulenz und Fitness-assoziierten Eigenschaften ausgewählter *E. coli* Mastitis- und Fäkalisolate wurden in vergleichenden phenotypischen Assays identifiziert und analysiert. Zur Untersuchung der bakteriellen Fitness in Milchdrüsensekreten wurde native Molke mit antimikrobiellen Eigenschaften von Rohmilch genutzt. Dabei stellte sich heraus dass *E. coli* Fäkalisolate eine heterogene Gruppe bilden. Innerhalb dieser Gruppe wiesen einige Isolate eine verminderte Überlebensrate auf. Andere Fäkalisolate zeigten eine höhere Überlebensrate, ähnlich der Überlebensrate von Mastitiserregern. Im Gegensatz zum ihrem grundsätzlich guten Überleben in Molke zeigten Mastitisisolate keine einheitlichen phänotypischen Merkmale bei Wachstum mit 1) Lactose als einziger Kohlenstoffquelle, 2) Eisenlimitierung, oder 3) unter Einfluss von lingualem antimikrobiellem Peptid (LAP), einem bedeutenden Defensin der Wirtsantwort im Euter. Die verminderte Fähigkeit in Milchdrüsensekreten zu überleben korrelierte mit der konzentrationsabhängigen Überlebensfähigkeit in Gegenwart von LAP. Dies lässt vermuten dass eine Anpassung der Bakterien an die Lebensbedingungen in der bovinen Milchdrüse der Resistenz gegenüber Defensinen (u.a. LAP) bedarf.

Den Mastitis-isolaten *E. coli* 1303 und ECC-1470 fehlen diverse Virulenzgene die bereits mit Mastitis assoziiert werden konnten. Um zu bestimmen ob Unterschiede in der Genexpression beider *E. coli* Isolate dazu beitragen Mastitis auszulösen, wurden Transkriptomanalysen durchgeführt. Dabei sollten vor allem Kandidatengene bestimmt werden, die an der Wirt-Pathogen-Interaktion beteiligt sind oder zur bakteriellen Fitness oder Virulenz der Erreger beitragen.

Auf der Basis von DNA Microarrays wurde die Genexpression von *E. coli* 1303 und ECC-1470 in Gegenwart von immortalisierten Zellen des bovinen Milchdrüsenepithels (MAC-T) bestimmt. Zusätzlich wurde die Fähigkeit zur Zelladhäsion und Internalisierung beider Isolate untersucht. Die bakterielle Transkriptionsantwort in Gegenwart der Wirtszellen ergab, dass Erreger und Wirtszellen eher um den Bedarf an Nährstoffen und Sauerstoff konkurrierten, anstatt deutliche Anzeichen der

Zelladhäsion oder Invasion zu zeigen. Beide Isolate nutzten vornehmlich Fitnesseigenschaften, die auch bei der Besiedlung des Darms als dem primären Habitat von *E. coli* verwendet werden.

In dieser Studie wurde außerdem die Genexpression von *E. coli* 1303 und ECC-1470 in Reaktion auf Molke aus Rohmilch mittels Gesamt-Transkriptom-Sequenzierung (RNA Seq) untersucht. Die Transkriptomanalyse ergab keine wirklich deregulierten virulenz-assoziierten Gene in einer der beiden *E. coli* Mastitis Isolate. Ferner konnten Eigenschaften identifiziert werden, die zum Wachstum und Überleben in nativer Molke beitragen. Die Fähigkeit, Citrat zu verwerten, begünstigt das erfolgreiche Überleben in Milchdrüsensekten und stellt einen wichtigen Fitnessfaktor dar. Unsere Transkriptomdaten bestätigen dass Lactoferrin nur geringen Einfluss auf das Wachstum, von *E. coli* in Milchdrüsensekreten, hat. Die Expression bakterieller Determinanten, die an der Aufnahme von Eisen beteiligt sind, wurde herunterreguliert. Dies lässt darauf schließen dass Eisenaufnahme in den ersten Stunden der Kolonisierung durch die Erreger keine essentielle Fitnesseigenschaft darstellt. Vermutlich reicht die intrazelluläre Menge an Eisen aus, um eine zeitweise Unabhängigkeit von extrazellular verfügbarem Eisen zu ermöglichen. Diese These konnte durch unsere Transkriptomdaten gestützt werden und stellt eine wichtige Entdeckung in Bezug auf die Verfügbarkeit von Eisen während der Kolonisierung der Milchdrüse dar. Unsere Daten zeigen, dass die Resistenz gegenüber extrazellulärem Stress durch oxidative Bedingungen und Defensine des Wirtes von großer Bedeutung für das bakterielle Überleben in Milchdrüsensekreten ist.

Die vorliegende Thesis befasst sich mit wichtigen Aspekten der Wirt-Pathogen-Interaktion und der Anpassung an die antimikrobiellen Bedingungen während der Kolonisierung der Milchdrüse als Ort der Infektion.

II. INTRODUCTION

This study presents the identification and characterization of virulence-associated traits of *E. coli* bovine mastitis isolates.

II.1. Epidemiology of bovine mastitis

Bovine mastitis is defined as an inflammation of the mammary gland tissue caused by microorganisms, usually bacteria that have overcome the cow´s immune defense. It is induced when the pathogens enter the udder, multiply and produce metabolites or toxins that cause harm to the mammary gland tissue. Damaged tissue allows for increased vascular permeability. This results in altered milk composition accompanied by a reduction in milk yield (Hill, 1994). Both changes may differ vastly as their extent depends on the severity of the inflammatory response (Kitchen, 2009; Seegers et al., 2003; Pyörälä, 2003), mainly influenced by the causative pathogen (Zadoks et al., 2011). A broad variety of organisms has been identified as potential mastitis pathogens and is distinguished into either major or minor pathogens. The main mastitis-causing pathogens are *Escherichia coli*, *Streptococcus uberis* and *Staphylococcus aureus*. These bacteria have been termed major pathogens because of their association to clinical mastitis (CM). In contrast, other bacteria may be present in the udder and often have an overall beneficial effect by protecting against infection caused by major pathogens. These bacteria produce natural anti-bacterial substances or interfere with the growth of major pathogens and are thus termed minor pathogens. Because of their complex interaction with the mammary gland these minor pathogens can contribute to increased somatic cell counts (SCCs) and thus to the incidence of sub-clinical mastitis (SCM), but they usually do not cause CM.

II.1.1. Severity and duration

The course of mastitis is either acute, i.e. severe and sudden in onset, or chronic, which is defined as a long-developing syndrome that worsens over months and results in development of fibrous tissue (Gröhn et al., 1990). Usually mastitis is classified according to its pathogenesis in either CM or SCM, which is considered the most prevalent form of mastitis (Gianneechini et al., 2002; Akers, 2002).

CM shows visible signs which are further distinguished in mild and severe signs. Flakes or clots in the milk and a discoloration of the teat of the infected udder quarter which may have slight swelling are mild signs. Severe signs are abnormal watery secretion as well as a hot and swollen udder. More systemic symptoms are fever, rapid pulse, loss of appetite, dehydration, depression and fatal consequences.

On the contrary, SCM is not accompanied with visible signs of an infection and both, milk and udder, appear normal although stagnation in milk has been reported (Mungube et al., 2005). Therefore SCM can be harder to detect although the SCCs in the milk increase likewise in CM. To date, the SCC

proved to be the most useful diagnostic technique to detect the presence and occurrence of bovine mastitis and especially for bovine subclinical mastitis (Schukken et al., 2011).

Generally, bacteria associated to CM have been termed major pathogens (Djabri et al., 2002) whereas minor pathogens do not usually cause clinical forms of the disease but are associated to SCM due to increased SCCs. These bacteria are meant to be present in the udder and often have an overall beneficial effect on protection from infection by major pathogens, due to the production of natural anti-bacterial substances, induced leukocytosis and competitive growth with other bacteria (Reyher et al., 2012).

II.1.2. Origin of infection

Mastitis causing pathogens have been broadly classified into either environmental or contagious pathogens (Radostits et al., 2007).

Contagious pathogens are meant to be intramammary survivors and are spread from cow to cow. Environmental pathogens originate from bedding materials, manure and soil and are considered to be opportunistic invaders with no specialized survival properties. This view becomes obsolete, when taking into account that mastitis causing *E. coli*, which are considered environmental pathogens, are indeed able to persist for prolonged periods within the udder and also cause either chronic or recurrent forms of mastitis (Almeida et al., 2011; Döpfer et al., 2001 and 2000). Hence, a spread from one host to another due to inappropriate post-milking teat disinfection is most likely. Based on the situation that environmental mastitis pathogens are present in the housing and bedding, they can easily be transferred during milking or between milkings when the cow is foraging or lying down.

Physiology and Invasion of the Udder

A basic knowledge of the bovine udder anatomy and physiology allows for a better understanding of mastitis development (**Figure 1**). The histologically divided quarters of a bovine udder are composed of a teat cistern, gland cistern, milk ducts and surrounding glandular tissue. This secretory tissue includes millions of microscopic sacs, designated as alveoli and lined by milk-producing epithelium, which is supplied with nutrients by surrounding blood vessels. Muscle cells encircle each alveolus and squeeze the milk to the milk ducts and further through the teat canal and duct during milking. In between the milkings, milk accumulates in alveolar spaces, milk ducts and gland cistern.

Bacteria might either invade the udder through lesions, as teat damage is known to increase susceptibility of mastitis, or more frequently breach the teat canal in several ways (Schroeder, 2010). Between milkings bacteria multiply inside teat duct and are introduced into the gland cistern via the teat canal by their motility properties or pressure placed on the teat end caused by physical movement of the cow. Another opportunity is that bacteria may be propelled into or through the teat canal during machine milking.

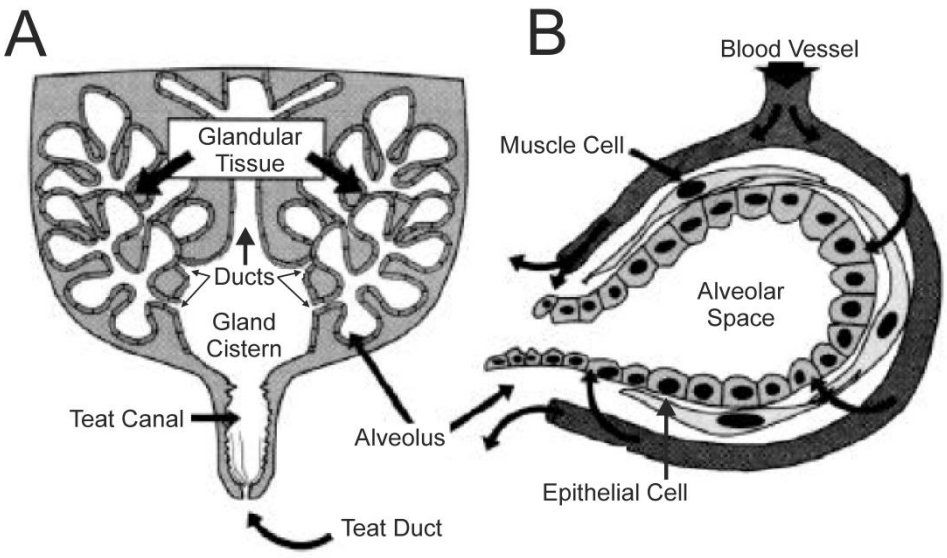

Figure 1: Scheme of the mammary gland modified from Schroeder, 2010. (A) Teat with gland cistern, milk ducts, and secretory glandular tissue. (B) Alveolus encircled by muscle cells and blood vessel.

II.1.3. Factors increasing mastitis susceptibility

The susceptibility to mastitis increases in dependence of various genetic and physiological ´cow factors´ as well as environmental factors (Hopster et al., 1998; Waller, 2000). Mastitis is therefore designated a multifactorial disease.

Environmental or Management factors

There is rarely an infection known unaffected by the environment. For instance the season affects the susceptibility to mastitis and there is consistency in the literature that CM occurs more frequently within the winter months (Steeneveld et al., 2008; Olde Riekerink et al., 2007).

Another factor, that is even more important, is poor hygiene which is directly correlated to infectious diseases and increases the risk of mastitis development (Schreiner and Ruegg, 2003). Intensive livestock breeding of dairy cattle implies high cow densities per unit and bad ventilation, and also the selection of bedding materials may facilitate bacterial growth (Ericsson Unnerstad et al., 2009). Given that stables are an environment which promotes bacterial spread, milking equipment might also be contaminated itself and enables bacteria to invade the udder (Hovinen and Pyörälä, 2011; Hässig et al., 2011). Moreover, dairy cattle suffering from stress generated by inappropriate human handling is known to be more susceptible to mastitis (Breuer et al., 2003). As a result it is unquestionable that management practices have been associated to CM (Parker et al., 2007; Barnouin et al., 2005; Barkema et al., 1999). Accordingly, the management of dairy herds is a comprehensible factor to lower the risks and provide more sustainable protection protocols to mastitis. Hence, there are still various anthropogenic factors to consider.

Cow factors

Besides environmental factors there are cow-specific factors to consider. The cow factors are defined by the cow´s immune response and genetic makeup and may conclude the cow´s age, SCC, parity, lactation stage, nutritional and metabolic status, CM history and genetic resistance. Selective breeding, focused on enhanced milk production, causes metabolic stresses relative to an increased milk yield and can be correlated to compromised mastitis resistance (Waller, 2000; Seegers et al., 2003). It is a known fact that cows with high milk yield are more likely to develop CM rather than cows with less milk yield (Gröhn et al., 2004).

Pathogens may infect cows both during the dry period and in lactation. The lactation stage has significant impact on the cow´s susceptibility to mastitis (Rinaldi et al., 2008; Mallard et al., 1998; Shafer-Weaver et al., 1996). It has been reported that the risk of developing CM is highest in early lactation (Steeneveld et al., 2008). Contrary, the risk of SCM increases with days in milk (Busato et al., 2000). Within the periparturient period of the lactation stage, the cow´s defense mechanisms are impaired and therefore the cow is at higher risk of developing mastitis with impact on subsequent intramammary infections (Breen et al., 2009; Oliver and Sordillo, 1988; Kehrli et al., 1989). It is known that multiparous cows show higher incidence of mastitis (Rajala-Schultz et al., 1999) and there is evidence that increasing parity correlates with increased susceptibility (Steeneveld et al., 2008).

Both, environmental and cow factors, are interdependent, whereas the relative impact of each factor is considerably influenced by the causative pathogen (Zadoks et al., 2011; Djabri et al., 2002).

II.1.4. Host defense mechanisms and pathogen recognition

The bovine mammary gland is protected by several defense mechanisms at the anatomical and cellular level. At the anatomical level the teat end is considered to be the first line of defense against invading bacteria that cause mastitis. The sphincter muscles at the end of the teat ducts should be tightly closed between milkings and thus inhibit bacterial penetration. The teat canal is lined with a waxy antimicrobial coating termed keratin, which functions as a physical barrier for invading bacteria (Nickerson, 1987; Treece et al., 1966). Both, dysfunctional sphincter muscles and diminished keratin lining of the teat canal have been independently shown to increase the risk bacterial invasion and colonization (Lefcourt, 1982; Capuco et al., 1992).

Bacteria that have overcome the anatomical defense mechanisms are then confronted with the bovine immune system. The mammary immune factors have been characterized into cellular and soluble components. At the cellular level we know about the functions of leukocytes which can be further distinguished into neutrophils, macrophages and lymphocytes. The cellular and soluble factors and their biological functions are listed in **Table 1** (Sordillo and Streicher, 2002). These defense mechanisms are further differentiated into innate immune and specific immune responses.

Innate immune response

The innate immune response is non-specific and rapidly eliminates bacteria before the specific immune system is activated. Abnormalities, e.g., changes in the milk composition do not occur. The non-specific responses are mediated by the physical barrier of the teat end, macrophages, neutrophils, natural killer cells, and particular soluble factors (Sordillo and Streicher, 2002). The soluble factors of the innate immune response include the bacteriostatic complement factors defensins, lysozyme, lactoferrin and cytokines.

The complement system represents the first defense line of innate immunity. The function of the mammalian complement system is to maintain homeostasis by recognition and removal of damaged or modified self-material and pathogenic microbes (Zipfel et al., 2013). However, complement is also of importance in the specific immune response. The complement system is composed of plasma proteins, which are also present in milk and mainly coat the surface of a pathogen for its recognition by cells of the specific immune system. During innate immune response the complement accounts for lysis of Gram-negative bacteria. Among these, *E. coli* has been reported to be sensitive to lysis by complement (Korhonen et al., 2000).

Additionally, the defensins, also referred to as antimicrobial peptides (AMP), are an evolutionarily conserved component of the innate immune response. Defensins are diverse oligopeptides (<100 amino acids), contributing to the antimicrobial action of granulocytes, mucosal and epithelial host defense (Ganz, 2003; Peschel and Sahl, 2006).

Of the soluble mammary gland defense components lactoferrin is a predisposing antimicrobial protein reported to increase in concentration when an inflammation occurs (Sordillo et al., 1987). Lactoferrin contributes to neutralization of cytotoxic effects mediated by lipopolysaccharides (Pecorini et al., 2010). Moreover, the main function of lactoferrin is to inhibit the growth of certain bacteria by binding of iron, an essential factor required for bacterial growth. This growth hindering effect of lactoferrin has already been reported for Gram-negative bacteria such as *E. coli* in ruminants (Chaneton et al., 2008; Rainard, 1986).

Cytokines are immunomodulating molecules that account for cell signaling. They are produced after antigen detection by certain cells of the immune system, mainly by leukocytes. The different cytokines (e.g. TNFα and IL1β) have a matching cell-surface receptor thus subsequently affecting intracellular signaling cascades and consequently mediating alterations of cellular functions. **Figure 2** shows the involvement of the inflammatory cytokines TNFα and IL1β in the immune response on putative mammary pathogenic *E. coli* (Shpigel et al., 2008) as possibly induced by lipid A recognition by the TLR4-MD-2 receptor complex (Maeshima and Fernandez, 2013). However, the specific effect of a particular cytokine depends on both, the cytokine´s and its receptor´s abundance followed by the activated downstream signaling cascade which can vary in different cell types. In general cytokines are considered redundant, in that many cytokines appear to share similar functions. These might include either direct regulation of genes or regulation by their transcription factors and further result in production of other cytokines, receptor expression for other molecules, or suppression of their own effect by feedback inhibition. Certain cytokines also participate in the specific immune response.

Specific immune response

If the infecting bacteria evade the innate immune response or are not completely eliminated, the specific or acquired immune response is triggered.

In contrast to the innate immune response the specific immune system needs to recognize the pathogens by specific antigens and therefore it takes time to be prepared. Once a specific response is created, due to the immunological memory, the immunity state is quickly established, intensive and enduring when the same antigen is recognized again. This results in a more effective elimination of the pathogen. Thereby, the most important soluble effectors of the specific immune response are antibodies produced by B lymphocytes after antigen recognition. The advantages of the acquired immune response are for example used as basis for vaccination.

Also in the mammary gland the innate and the acquired immune system are coordinated in synergy to prevent mastitis.

Table 1: Cellular and soluble defenses of the mammary gland

Cellular defense factors	Biological function	Immune system
Neutrophils	Phagocytosis and intracellular killing of bacteria; secretion of antibacterial factors	innate
Macrophages	Phagocytosis and intracellular killing of bacteria; antigen presentation in conjunction with MHC	innate & specific
Natural killer cells	Non-immune lymphocytes that secrete antibacterial Proteins upon activation	innate
T lymphocytes		
CD4C (T helper)	Production of immunoregulatory cytokines following antigen recognition with MHC class II molecules; memory cells following antigen recognition	specific
CD8C (T cytotoxic)	Lysis of altered or damaged host cells when complexed with MHC class I molecules; production of cytokines that can down-regulate certain leukocyte functions	specific
γδ T lymphocytes	Biological role in the mammary gland is speculative	specific
B lymphocytes		
Mature B cells	Display membrane-bound antibody molecules to facilitate antigen presentation; memory cells following antigen interactions	specific
Plasma cell	Terminally differentiated B lymphocytes that synthesize and secrete antibody against a specific antigen	specific

Soluble defense factors	Biological function	Immune system
Cytokines	Proinflammatory and immunoregulatory factors	innate
Complement	Bacteriolytic and/or facilitates phagocytosis	innate & specific
Lysozyme	Cleaves carbon bonds and disrupts bacterial cell walls	innate
Lactoferrin	Sequesters iron to prevent bacterial uptake; disrupts bacterial cell wall; regulates mammary leukocyte activity	innate
Antibodies		
IgG1	Selectively transported into mammary secretions; opsonizes bacteria to enhance phagocytosis	innate
IgG_2	Transported into secretions during neutrophil diapedesis; opsonizes bacteria to enhance phagocytosis	innate
IgA	Associated with the fat portion of milk; does not bind complement or opsonize particles; can cause agglutination, prevent bacterial colonization, and neutralize toxin	innate
IgM	Efficient at complement fixation, opsonization, agglutination and toxin neutralization; only opsonic for neutrophils in presence of complement	innate

Table modified from Sordillo and Streicher, 2002

Pathogen recognition

Rapid pathogen recognition is essential for activation of the innate and the specific immune response. Generally, immune cells as well as non-immune cells are able to sense pathogens by particular pathogen recognition receptors (PRR). These receptors can be displayed either on the surface or intracellularly, and recognize so called pathogen-associated molecular patterns (PAMPs). PAMPs are small molecular motifs, which are conserved within a class of microbes such as lipoteichoic acids in Gram-positive and LPS in Gram-negative bacteria (Kumar et al., 2011).

After binding to PAMPs, the PRRs subsequently initiate intracellular signaling cascades or directly promote the attachment, ingestion and destruction of the pathogens. **Table 2** shows a general view of these pathogen recognition receptors. Additionally, two lymphocytes types of the specific immune system (also referred to as memory B cells and memory T cells) are able of pathogen recognition when sensing a previously encountered antigen allowing for a rapid immune response (Sordillo and Streicher, 2002; Sordillo et al., 1997). Of the extracellular PRRs the so called Toll-like receptors (TLRs) have a key role in activation of the innate and specific immune response. For instance when a specific PAMP such as LPS interacts with its particular TLR it induces, via TNFα, NF-κB signaling and the MAP kinase pathway followed by the release of cytokines and elicitors that will pass on the TLR activation signal to other immune cells (Doyle and O'Neill, 2006).

Table 2: Pathogen recognition receptors

Factor	Role
Innate Immunity	
CD14	Binds LPS. Membrane version is expressed on several cells including monocytes, macrophages, neutrophils, dendritic cells, and B cells. The soluble version may compete with mCD14 for LPS and is essential in the activation of non-mCD14 expressing cells, including epithelial and endothelial cells, by LPS.
PGRP	Expressed in differentiated, lactating epithelium where it binds and hydrolyzes peptidoglycans.
TLR2	Recognizes peptidoglycan and LTA from Gram-positive bacteria and lipoarabinomannan from mycobacteria. May form a heterodimer with TLR1 to recognize triacylated lipopeptides from Gram-negative bacteria and *Mycoplasma* or with TLR6 to recognize diacylated lipopeptides from Gram-positive bacteria and *Mycoplasma*.
TLR3	Detects double-stranded RNA.
TLR4	Recognizes LPS of Gram - bacteria, heat-shock proteins, fibrinogen, and polypeptides.
TLR5	Recognizes bacterial flagellin.
TLR9	Intracellular recognition of CpG-containing oligodeoxynucleotides (ODNs).
Acquired Immunity	
Fc Receptor	Expressed on macrophages, neutrophils, and natural killer cells and recognize antibodies of infected cells or pathogens.

Table modified from Aitken *et al.*, 2011

The recognition of a presumed mammary pathogenic *E. coli* (MPEC hereafter) is schematically shown in **Figure 2**. TLR2 and TLR4 are the most significant receptors during bacterial mastitis and are primarily activated in response to Gram-positive and Gram-negative infections, respectively (Aitken et al., 2011; Günther et al., 2011; Yang et al., 2008; Goldammer et al., 2004).

Notably, even though both *S. aureus* and *E. coli* are able to trigger TLR2 and TLR4, only *E. coli* is capable of inducing NF-κB signaling in mammary gland epithelial cells followed by quick induction of TNF-α. It is still not known whether the diminished NF-κB activation potential in immune relevant cells is a molecular defect, or depends on virulent traits of the particular pathogen, associated with subclinical mastitis.

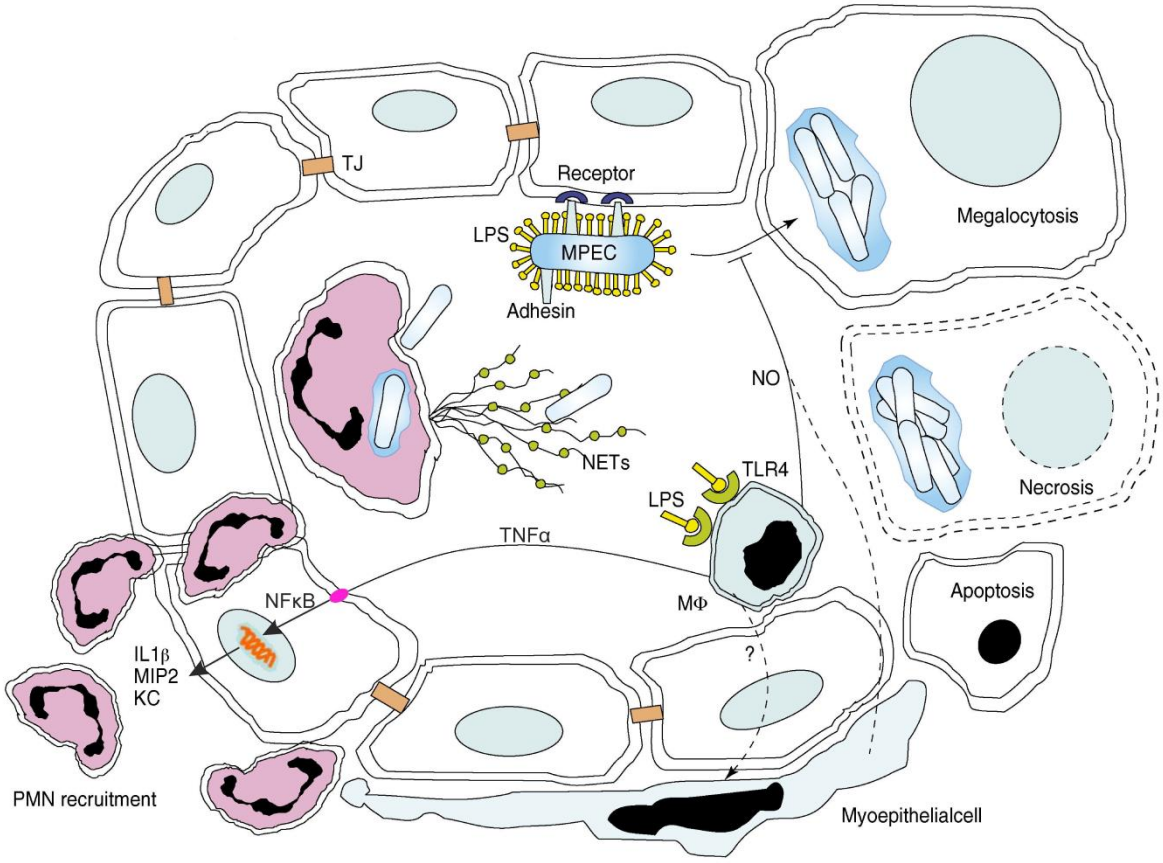

Figure 2: Pathophysiological scheme of presumed mammary pathogenic *E. coli* (MPEC) replicating in the mammary alveolar space, modified from Shpigel et al., 2008. LPS/TLR4 signaling on alveolar macrophages (MΦ) elicits production of inflammatory cytokines (TNFα and IL1β) and chemokines (KC and MIP2), resulting in recruitment of blood neutrophils (PMN) trafficking across the polar alveolar epithelium (TJ, tight junction) into the alveolar space. Recruited neutrophils are killing the bacteria by phagocytosis and neutrophil extracellular traps (NETs). Bacterial epithelial invasion is abrogated by LPS/TLR4 signaling on MΦ possibly mediated by nitric oxide (NO) produced by MΦ and myoepithelial cells. Epithelial invasion by bacteria induce epithelial megalocytosis, necrosis and apoptosis.

II.2. *Escherichia coli*

Escherichia coli (*E. coli*) represents from an evolutionary point of view a comparably young bacterial species linked to the appearance of animals. Usually, a commensal resident of the intestinal microflora of warm blooded animals, *E. coli* can become pathogenic by acquisition of virulence factors (VFs) (Hacker et al., 2003). Specific pathotypes of *E. coli* are defined by successful combinations of VFs, often located on pathogenicity islands (PAIs) or smaller inserts. Pathotypes are capable of causing a broad spectrum of diseases in healthy individuals (Kaper et al., 2004). Besides specific pathotypes (**Table 3**), the major categories are: intestinal pathogenic *E. coli* (IPEC), extraintestinal *E. coli* (ExPEC), depending on the site of infection, and commensal *E. coli* (Dobrindt, 2005; Köhler and Dobrindt, 2011). Notably, *E. coli* pathotypes cannot always be unambiguously grouped by phylogeny. They often arose in parallel evolution and were spread polyphyletically (Reid et al., 2000). *E. coli* strains can be classified according to their phylogeny into clonal groups (A, B1, B2, C-I to C-V, D E and F) and cryptic clades (Clermont et al., 2000 and 2013; Walk et al., 2009). Moreover, *E. coli* strains can be distinguished by their serotype, defined by the combination of their surface antigens LPS (O antigen), flagellin (H antigen) and capsule (K antigen) (Kauffmann, 1965). Serotyping is an approved diagnostic tool which allows to associate *E. coli* strains of a specific serotype to certain clinical manifestations. However, the classification by serotype does not allow conclusions concerning virulence (Jacks and Glantz, 1967). Instead the serotype correlates to the strains phylogeny and often provides valuable information on its pathotype (Iguchi et al., 2008). Altogether, the different pathotypes, high phylogenetic diversity and various serotypes of *E. coli* result from substantial genome plasticity.

Table 3: Intestinal und extraintestinal *Escherichia coli* pathotypes.

Intestinal pathogenic *E. coli* (IPEC)	Extraintestinal pathogenic *E. coli* (ExPEC)
Adherent invasive *E. coli* (AIEC)	Avian pathogenic *E. coli* (APEC)
Diffusely adherent *E. coli* (DAEC)	Meningitis-associated *E. coli* (NMEC)
Enterotoxigenic *E. coli* (ETEC)	Septicemia-associated *E. coli* (SEPEC)
Enteropathogenic *E. coli* (EPEC)	Uropathogenic *E. coli* (UPEC)
Enteroinvasive *E. coli* (EIEC)	putative *Mammary pathogenic E. coli (MPEC)*
Enteroaggregative *E. coli* (EAEC)	
Enterohemorrhagic *E. coli* (EHEC)	

II.2.2. *E. coli* genome structure and plasticity

The *E. coli* genome is varying from approximately 4.6 to 5.5 × 10^6 bp in size. It can be distinguished into a core genome, present in all *E. coli* strains, and a strain-specific dispensable genome also referred to as flexible gene pool. Obviously, the core genome comprises the so called housekeeping genes, encoding for proteins of translation, transcription and replication, as well genes of the basic metabolism. Moreover, it can be suggested that some genes of the core genome are important in the lifestyle within the gut as the main *E. coli* habitat. Once estimated with about 3,100 genes, more recent calculations report only about 2,000 genes of high homology comprised by the core genome of *E. coli* (Dobrindt et al., 2003; Touchon et al., 2009). On the other hand, the flexible gene pool (16,000 genes) accounts for the different genome sizes of *E. coli* and thereby comprises genes that are required for adaptation to often changing environmental conditions provided by the habitat. These genes are often localized on mobile genetic elements, such as plasmids, prophages, transposons or genomic islands (GEIs). GEIs may serve as integration hotspots for different mobile genetic elements. They are discrete genetic units of 10-200 kb, flanked by direct repeats and insertion sequences and frequently associated with tRNA-encoding genes. Their GC-content differs from that of the core genome. Additionally, GEIs comprise genes coding for integrases and transposases, which facilitate the insertion into the chromosome of recipient *E. coli* strains. If a GEI comprises virulence genes and if this GEI is present in the genome of a pathogenic *E. coli*, but absent in non-pathogenic strains, this GEI is referred to as a pathogenicity island (PAI). Both, the core genome and the dispensable genome constitute the *E. coli* pangenome (approximately 18,000 genes) (Dobrindt et al., 2003 and 2004; Hacker et al., 1990, 1997 and 2003; Leimbach et al., 2013; Medini et al., 2005; Tenaillon et al., 2010, Touchon et al., 2009).

The substantial genome plasticity makes *E. coli* a highly versatile species which is able to constantly alter its genome content by horizontal gene transfer (HGT) and deletion events in order to provide adaptation, fitness and competitiveness to different growth conditions and habitats .

II.2.3. ExPEC: Commensal *E. coli* with increased potential to cause extraintestinal disease

The acronym ExPEC designates certain *E. coli* strains that cause extraintestinal infections (EIs). Hence, a specific *E. coli* strain would be defined as ExPEC if it exhibits enhanced virulence in an appropriate extraintestinal infection model or if harboring multiple extraintestinal VFs (**Table 4**). Isolation of an *E. coli* strain from an extraintestinal infection does not *per se* determine an ExPEC, since also commensal strains of *E. coli* are able to cause EI when the host is compromised. Likewise the specific pathotypes of *E. coli* (**Table 3**), the major category ExPEC is defined by successful combinations of VFs, depending on the number, type and synergistic effect of these VFs (Russo and Johnson, 2000; Kaper et al., 2004; Köhler and Dobrindt, 2011).

Table 4: ExPEC virulence factors as illustrated by Köhler and Dobrindt, 2011

Functional category	Virulence factor
Adhesin	Type 1 fimbriae (Fim)
	P fimbriae (Pap/Prf)
	S/F1C fimbriae (Sfa/Foc)
	N-Acetyl D-glucosamine-specific fimbriae (Gaf)
	M-agglutinin (Bma)
	bifunctional enterobactin receptor/adhesin (Iha)
	afimbrial adhesin (Afa)
	temperature sensitive hemagglutinin (Tsh)
Invasin	invasion of brain endothelium (IbeA)
Iron acquisition	siderophore receptor IreA
	aerobactin (Iuc)
	yersiniabactin (Ybt)
	salmochelin (Iro)
	periplasmic iron binding protein (SitA)
Toxins	alpha-hemolysin (HlyA)
	cytolethal distending toxin I (CDT 1)
	cytotoxic necrotizing factor 1 (CNF-1)
	colibactin (Clb)
	serine protease autotransporters Sat, Pic
Protectins	group II capsule incl. K1 capsule
	conjugal transfer surface exclusion protein (TraT)
	outer membrane protease T (OmpT)
	increased serum survival (Iss)
	colicin V (Cva)
Others	D-serine deaminase (DsdA)
	maltose and glucose-specific PTS transporter subunit
	flagella

It seems clear that the flexible *E. coli* gene pool serves the purpose to acquire and discard genes as a result of selective pressures of the habitat. This opens up a rather new perspective of discriminating pathogens from commensals. While IPEC strains can be reliably discriminated from ExPEC or commensal *E. coli* based on genome content and phenotypic traits, an unambiguous distinction between ExPEC and commensals proves to be difficult (Leimbach et al., 2013). This is easy to understand as ExPEC originate from the microflora of healthy individuals by acquisition of novel traits. Or changing the perspective: Commensals constitute as reservoir of *E. coli* that can become ExPEC.

In terms of commensalism advantageous traits are referred to as colonization and fitness factors, whereas traits contributing to pathogenicity have been designated virulence factors (VFs). The literature refers to VFs as molecular mechanisms expressed by pathogens enabling them to thrive in pathogenesis (Donnenberg, 2002). VFs might facilitate the colonization of a niche in the host (e.g. the teat duct or the urinary tract) including the adhesion to host cells or actually mediate immunoevasion (evasion of the host's immune response), e.g. by invasion/entry into host cells. They might also enable nutrient acquisition from the host or cause inhibition of the host's immune response also referred to as immunosuppression. As PAIs may carry multiple virulence-associated gene clusters, its acquisition might turn a before benign *E. coli* strain into a pathogenic one in a single step (Dobrindt et al., 2004).

Anyhow, it is an ongoing discussion whether particular traits should be considered VFs or fitness factors depending on their contribution to either one or being involved in both ExPEC virulence and commensal fitness. For instance Leimbach et al. exemplified prevalent VFs of ExPEC that are also found in commensal *E. coli* (**Table 5**). Furthermore, they pointed out that commensal *E. coli* of phylogroups B2 and D resemble typical ExPEC with regard to the prevalence of their virulence- or fitness-associated traits. This is supported by data suggesting that extraintestinal virulence emerged as a coincidental by-product of commensalism (Le Gall et al., 2007; Tenaillon et al., 2010). And, of course, it seems convincing that some of these virulence-associated traits (e.g. adhesins) are important not only for pathogens but also for commensals within the gut. The odd situation that many previously described virulence-associated traits were announced VFs, although contributing to commensalism, may be due to the focus on pathogens in former studies. Notably, in this study the traits contributing to bacterial virulence and survival within the bovine udder, which are not known to be indispensable for ExPEC virulence, will be announced virulence-associated traits hereafter.

Nevertheless, there some indications may be helpful to distinguish between ExPEC and commensals: Commensals are highly adapted to a particular niche, like a specific gut region. It seems natural that characteristics of a rather persistant commensal depend on the site of the habitat, while a transient pathogen may be not that well-adapted to this niche (Abraham et al., 2012). The acquisition of novel

genes as well as genome reduction, are reflected by variable genome sizes. However, the genome size of commensal *E. coli* has been reported to be usually smaller than that of pathogenic *E. coli* variants (Chaudhuri and Henderson, 2012; Bergthorsson and Ochman, 1995). Another opportunity might be the characterization of source-specific alleles (e.g. of *iss* or genes of extracellular polysaccharides) that presumably differ and could improve discrimination of ExPEC and commensal *E. coli* (Johnson et al., 2008a; Duda et al., 2013). As afore mentioned VFs are often localized on PAIs or plasmids, some commensals might be distinguished from ExPEC according to their plasmid content (Dufour et al., 2011), although mobile elements should not account as unambiguous marker. The *pks* island for example, a designated PAI, was associated with multiple ExPEC-associated virulence genes identified in an especially high-virulence subset of phylogroup B2 (Johnson et al., 2008b). The same authors found that ExPEC and commensal *E. coli* differ in their antimicrobial susceptibilities and plasmid replicon possession (colicin plasmid) suggesting that in ExPEC, multi drug resistance (MDR) is frequently associated with plasmids (Johnson et al., 2012). In summary: We know that improved adaptability and competitiveness may promote intestinal colonization as well as extraintestinal infection by *E. coli* (Leimbach et al., 2013). As a consequence extraintestinal virulence and intestinal fitness traits overlap. In the era of genomics discrimination of ExPEC and commensal *E. coli* should be further improved by either 1) extended studies on the prevalence of source-specific alleles or 2) assessment of gene regulation of the traits involved in both fitness and virulence related processes.

Table 5: Fitness and virulence traits of ExPEC adapted from Leimbach et al., 2013

Trait	Example	Role during infection	Role during commensalism or in secondary habitat
Adhesins	Type 1 fimbriae	Adhesion, niche tropism, biofilm formation	Adhesion, niche tropism, biofilm formation
Siderophore receptors	Yersiniabactin receptor Salmochelin receptor irgA homolog adhesin (Iha)	Iron acquisition, adhesion, invasion, biofilm formation	Iron acquisition, adhesion, biofilm formation
Extracellular polysaccharides, cellulose, capsule, LPS	Capsule, cellulose, LPS	Serum resistance, protection against immune response; interaction with eukaryotic cells	Protection against predation, desiccation, intestinal colonization
Toxins	α-Hemolysin	Cell/tissue destruction, release of nutrients	Signaling
Flagella		Motility/chemotaxis	Motility/chemotaxis
Metabolic traits	Utilization of D-serine, fructooligosaccharides	Growth advantage, niche colonization	Growth advantage, niche colonization

II.2.4. Virulence factors of mammary pathogenic *E. coli*

This study focuses on virulence-associated traits of *E. coli* strains capable of causing bovine mastitis. Therefore, typical VFs of ExPEC will be discussed in relation to MPEC.

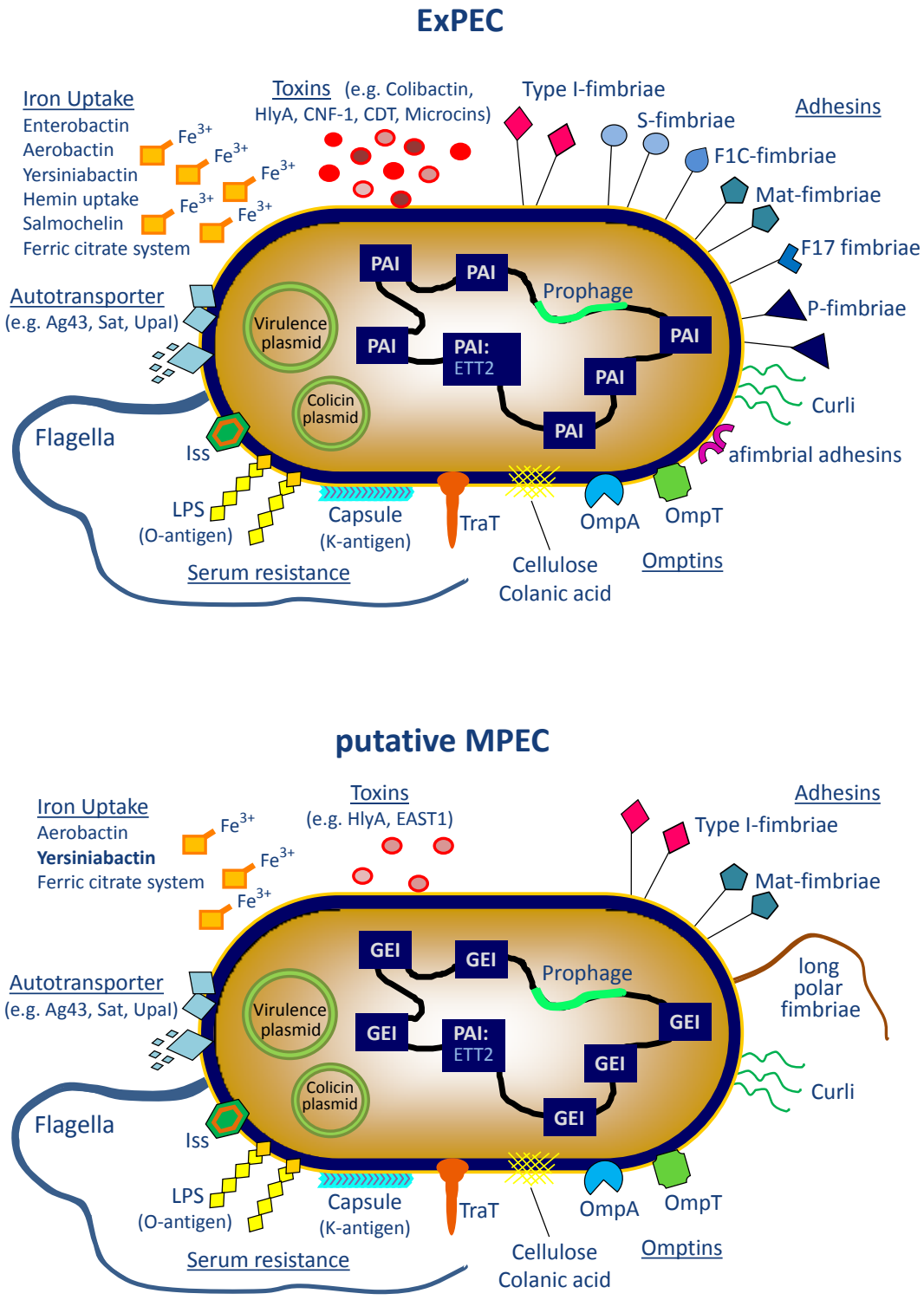

Figure 3: Scheme of different typical VFs of extraintestinal pathogenic *E. coli* (ExPEC) in comparison to a mammary pathogenic *E. coli* (MPEC) and its suggested VFs.

It was mentioned before (section II.2.2), that alterations of the *E. coli* genome content due to HGT and genome reduction can result in adaptation, improved fitness and competitiveness to different growth conditions and niches. Thus, a PAI conferring virulence traits might turn a benign *E. coli* strain into a pathogenic one in a single step (Dobrindt et al., 2004). A study conducted by Dufour et al. reports on the presence of GEIs in the bovine mastitis *E. coli* strain P4. The complete characterization of one GEI revealed genes that clearly distinguish *E. coli* P4 from the *E. coli* K-12 strain MG1655 and which are present in other pathogenic *E. coli* strains (Dufour et al., 2011). Indeed, knowledge of the presence or absence of GEIs or designated PAIs might allow us to distinguish between commensal *E. coli* or such with the ability to cause mammary gland infection (MPEC) as suggested by Shpigel et al. (Shpigel et al., 2008).

The Type Three Secretion System ETT2

The *E. coli* type three secretion system 2 (ETT2)-encoding island is a designated PAI, which is distributed among many *E. coli* (Ren et al., 2004). The 29.9 kb ETT2 PAI was first discovered in the genome of EHEC O157:H7 serotype strain EDL933 (Perna et al., 2001) and mainly associated with pathogenic *E. coli* (Makino et al., 2003; Miyazaki et al., 2002). Later, the ETT2 gene cluster was found either in total or in part in the majority of *E. coli* strains. However, according to its genetic structure it seems often to encode a non-functional secretion system (Ren et al., 2004; Perna et al., 2001). The intact ETT2 locus encodes for at least 35 genes, including *yqe*, *yge*, *etr*, *epr*, *epa*, *eiv*, etc., which are similar to genes included in PAIs of *Salmonella* (Cheng et al., 2012; Hansen-Wester and Hensel, 2001; Blanc-Potard et al., 1999; Galán, 1996). The presence of ETT2 was previously associated with bacterial invasion and intracellular survival of MNEC and was therefore believed to be involved in the pathogenesis of extraintestinal infection (Yao et al., 2009). In general, the type three secretion system (T3SS) is exclusively present in Gram-negative bacteria and is involved in the transport of bacterial virulence-associated proteins across the bacterial membranes, but also across the host cell barriers directly into the host cell cytoplasm (Cornelis, 2002; Lee, 1997). On the contrary, the specific role of the ETT2-encoded proteins and their functions in pathogenesis remains to be investigated in detail. Interestingly, a recent study on the prevalence and isoforms of ETT2 revealed a significantly higher presence of ETT2 (86 %, n= 92) among *E. coli* isolates of porcine origin than that (47 %, n= 76) of bovine mastitis origin. Even more interesting is the correlation of the presence of the intact ETT2 in the isolates from cases of porcine edema and/or diarrhea. In contrast, the majority of isolates from bovine mastitis carried corrupted EET2 isoforms and showed no distinct association with other VFs, e.g., the presence/absence of heat-labile enterotoxin(LT1), heat-stabile enterotoxin(ST2), cytotoxic necrotizing factor type 2(CNF2), pili (Tra), catalase-peroxidase(HPI) and hemolysin(Hly) (Cheng et al., 2012).

Adhesins

The VFs enabling the bacteria to adhere to host epithelial cells are referred to as adhesins. Adhesins can be divided into fimbrial and afimbrial adhesins. Fimbriae (also pili) are thin, rod shaped fibers composed of different protein subunits (Sauer et al., 2000; Schilling et al., 2001).

Type 1 fimbriae

The type 1 fimbriae represent the most prevalent fimbrial-type in *E. coli* and are encoded by the *fim* gene cluster comprising nine *fim* genes. Among them is *fimH* which encodes for the adhesion and is located at the very tip of the fimbriae. The FimH subunit has been reported to mediate specific binding to α-mannoside structures on different animal host epithelial cells (Klemm et al., 1985; Johnson, 1991; Pourbakhsh et al., 1997). Additionally, binding to IgA, laminin and also to the CD 11 and CD 18 complex of leukocytes and macrophages was observed. Interestingly, the comparison of *fimH* genes of *E. coli* strains from bovine, avian and porcine clinical cases revealed a substantial homology (>99%) among *fimH* genes from the different animal species origins. Moreover, specific mutations were found, some of which were present more frequently in bovine or avian or porcine strains respectively (Vandemaele et al., 2004).

Long polar fimbriae

The long polar fimbriae (*lpf* gene cluster) were first discovered in *Salmonella typhimurium*. The designation reflects the observation of long fimbriae inserted at the poles of the bacterium upon expression of the fimbrial operon by a non-piliated *E. coli* strain (Bäumler and Heffron, 1995). Despite this, a fimbrial cluster of high sequence similarity to the *lpf* gene cluster of *Salmonella* and several IPEC strains has been identified in ExPEC. This operon exhibits homology regarding the nucleotide sequence and the genetic organization relative to the type I fimbrial gene cluster. Furthermore, the ExPEC *lpf* cluster was reported to be functional and involved in adherence and invasion to kidney epithelial cells(Ideses et al., 2005).

Regarding mastitis, the presence of the *lpfA* gene was associated with invasion of cultured bovine mammary gland epithelial cells by *E. coli* isolates from both, transient and persistent, mastitis cases (Dogan et al., 2012). A recent study determined the *lpfA* gene as one of the most prevalent VF genes detected in *E. coli* isolates from bovine mastitis (Blum and Leitner, 2013). This study found *lpfA* prevalent in 52 % of the mastitis isolates (n= 63) and 67 % of the environmental isolates (n= 24) investigated. Both studies support the idea of long polar fimbriae being an important factor in the pathogenesis of mastitis-causing *E. coli*. However, this is still a matter of speculation and needs to be reinforced by *in vivo* experiments as well as more extended studies on the prevalence of the *lpf* gene cluster.

F1C, P and S -fimbriae

Also distributed in ExPEC isolates are fimbriae of S-, F1C-, and P-type, which also possess an adhesin present at the tip mediating binding to sugar entities of certain host cell receptors (Johnson, 1991). In contrast to many other VFs, they are exclusively encoded on the bacteria´s chromosome (Hacker, 1992).These fimbriae were observed to contribute to the virulence of pathogenic *E. coli* strains, but they are not necessarily sufficient to cause disease (Mobley et al., 1994). However, P-fimbriae account for host responses resulting in inflammation (van den Bosch et al., 1993; Bergsten et al., 2005). The P-fimbriae are encoded by the *pap* gene cluster and are reported to be less often expressed in ExPEC compared to type 1 fimbriae expression (Hacker, 1992). There is evidence that P-fimbrial adhesins may be associated with the virulence of avian pathogenic *E. coli* (APEC) (Kariyawasam and Nolan, 2011).

The S fimbriae (encoded by the *sfa* gene cluster) and F1C fimbriae (encoded by the *foc* gene cluster) are members of the S-fimbriae superfamily of adhesins. S fimbriae mediate agglutination (mannose-resistant hemagglutination /MRHA) of human erythrocytes. The S and F1C fimbriae show specific binding to sialosyl oligosaccharide chains (Ott et al., 1988; Prasadarao et al., 1993). Studies on the prevalence of the S fimbriae among ExPEC strains reported greatly varying frequencies of the *sfa* genes reaching from 9.2 to 97 % in APEC strains and 50 to 100 % prevalence in human ExPEC isolates (Ewers et al., 2007; Moulin-Schouleur et al., 2006).

For S- and P-fimbriae, there is a merely slight prevalence (7-8 %, n= 155 and 160) in *E. coli* bovine mastitis isolates from Finland reported (Lehtolainen et al., 2003; Kaipainen et al., 2002). In contrast, a more recent study on bovine mastitis isolates found none of the isolates containing genes for F17a-A, intimin, P or S fimbriae (Ghanbarpour and Oswald, 2010). Notably, this would lead to the suggestion that mastitis pathogenesis does not require these particular adhesins.

F17 fimbriae

F17 fimbriae were mainly reported in pathogenic *E. coli* strains responsible for diarrhea or septicemia in cattle and sheep and more recently associated with APEC (Oswald et al., 1991; Lintermans et al., 1988; Le Bouguénec and Bertin, 1999; Stordeur et al., 2002). Although F17-positive APEC strains were pathogenic for chicken and caused characteristic lesions of avian colibacillosis (Stordeur et al., 2004), there is still doubt that the observed contribution is not an effect of other known VFs. F17 fimbrial-related genes were identified in other ExPEC, e.g. human UPEC and bovine SEPEC isolates (Martin et al., 1997; Le Bouguénec and Bertin, 1999) and were also detected in *E. coli* bovine mastitis isolates (20 %, n= 127) (Ghanbarpour and Oswald, 2010). Again, there is a contrast to the Finnish *E. coli* bovine mastitis isolates which showed a slight prevalence of F17 fimbriae (8-9 %, n= 155 and 160) (Lehtolainen et al., 2003; Kaipainen et al., 2002).

Mat fimbriae

The Mat (meningitis-associated and temperature regulated) fimbriae were originally identified in O18:K1:H7 NMEC isolates and are known to be encoded by the *mat* gene cluster (Pouttu et al., 2001). Later, expression of a structurally related fimbria was observed in both pathogenic and non-pathogenic *E. coli* strains and thus the name *E. coli* common pilus (ECP) was introduced (Rendón et al., 2007). Nowadays, we know that the *mat* gene cluster has an identical chromosomal location as well as an overall 98% DNA sequence identity across different pathovars and phylogenetic groups of *E. coli*. Therefore the *mat* gene cluster belongs to the so-called persistent genes that are present in nearly all isolates of a species (Touchon et al., 2009; Fang et al., 2005). To date, the Mat fimbriae were frequently observed to mediate binding to particular epithelial cells (Rendón et al., 2007; Lasaro et al., 2009; Saldaña et al., 2009; Avelino et al., 2010). Just recently EcpD/MatE was presumed to be responsible for the attachment to epithelial cells. This suggests the presence of a tip-associated adhesin like in case of other fimbrial adhesins (Garnett et al., 2012). Furthermore, Mat fimbriae are needed for biofilm formation by NMEC and UPEC (Lehti et al., 2010; Garnett et al., 2012). Given that Mat fimbriae are essential for the colonization of infant mice by the probiotic isolate *E. coli* Nissle 1917 (Lasaro et al., 2009), the Mat fimbria is considered rather an important colonization factor of *E. coli* than a virulence factor. However, although there is no relation to putative MPEC reported to date, the Mat fimbriae could be an advantageous factor in mastitis pathogenesis when facilitate binding to mammary epithelial cells.

Curli

Curli are thin, filamentous structures frequently displayed on the surface of ExPEC, which are encoded by the *csgBAC* operon. The adhesive subunit CsgA facilitates binding to serum proteins and proteins of the extracellular matrix (e.g. laminin, fibronectin, plasminogen), but also interacts with major histocompatibility complex molecules (Olsén et al., 1998 and 1989). *E. coli* mastitis isolates, deprived of mannose-sensitive and mannose-resistant adhesins, were able to produce curli fimbriae under aerobic and anaerobic conditions at room and higher temperature, which suggests that these adhesins may be involved in the pathogenesis of bovine mastitis (Karczmarczyk et al., 2008). Curli expression was detected in 55-57 % of the clinical mastitis isolates tested (Dyer et al., 2007; Olsén et al., 1989). However, phenotypic curli expression in clinical isolates did not affect recovery of the cows' milk yield to premastitis production levels. This suggested that the clinical severity of *E. coli* mastitis is more dependent on cow-related factors than on bacterial virulence (Dyer et al., 2007). Nevertheless, curli enable bacterial adherence to multiple cell lines, and curli fibers themselves interact with many host proteins and are potent inducers of the host inflammatory response (Barnhart and Chapman, 2006). The role of curli expression in *E. coli* pathogenesis during mastitis remains yet unclear.

Afimbrial adhesins

Structures that facilitate binding, but are not assembled into fimbria structures are designated afimbrial adhesins. The first reported afimbrial adhesin was that encoded by the *afa* gene cluster and was observed to mediate UPEC binding to epithelial cells of the urinary tract (Labigne-Roussel et al., 1984). Afa and the closely related adhesins from Dr adhesin superfamily and the mannose resistant non-fimbrial hemagglutinin(NFA) are expressed by ExPEC and IPEC from both human and domestic animals (Le Bouguénec and Bertin, 1999; Girardeau et al., 2003). Another study suggested Afa to be associated with EPEC (Keller et al., 2002), but *afa* genes were rarely (1 %) detected in *E. coli* isolates from bovine mastitis (Kaipainen et al., 2002). Similarly, other afimbrial adhesins have been associated rather with IPEC than ExPEC (e.g. CS6 and CS31A adhesins). The CS31A adhesion for example is mainly expressed by pathogenic *E. coli* strains also producing the F17 fimbriae which were only rarely detected (<1 %) in putative MPEC (Ghanbarpour and Oswald, 2010; Kaipainen et al., 2002).

Flagella

The flagella are long surface structures composed of polymerized flagellin subunits encoded by *fliC*. Flagella mediated motility and were shown to affect the pathogenesis of UTI caused by UPEC (Lane et al., 2005). Furthermore, flagella were reported to enhance colonization of the urinary tract by UPEC and enable ascending infections (Lane et al., 2007). Beside their impact on ExPEC pathogenesis, flagella have been associated with invasion into intestinal epithelial cells of domestic animals by IPEC (ETEC, EPEC and STEC) (Xu et al., 2013; Duan et al., 2012 and 2013; Girón et al., 2002; Murinda et al., 2004). Anyhow, flagella might provide the motility required by putative MPEC to ascend the milk ducts. Just recently, *fliC* was for the first time reported in context of mastitis; found to be present in 80 % of isolates from bovine mastitis (n= 20) (Silva et al., 2013). It has to be further elucidated whether *fliC* is expressed and thus impact mastitis or not.

Serum and complement resistance

E. coli strains that cause extraintestinal infections are challenged with anatomical barriers and early stages of host defense (section II.1.4). In order to resist to the host´s complement system during an infection bacteria might either utilize polysaccharides (e.g. K-antigens and O-antigens) or proteins (e.g. Omp or Iss). Additionally, bacteria have evolved different mechanisms to resist the serum killing by defensins (also AMP) (Peschel and Sahl, 2006).

Capsules; K-antigens

In the past, various functions have been assigned to cell coating capsular polysaccharides of different bacterial species. These include adhesion, transmission, resistance to innate host defenses, resistance to the host's adaptive immune response (e.g. complement-mediated killing) and intracellular survival (Roberts, 1996). The capsules or so called K-antigens are known to mediate

serum resistance and protection from complement by either inhibition of the complement activation cascade or due to steric effects and masking of the cell surface. Either way, the mediated resistance is likely to involve a number of cell surface structures which contribute to the overall effect (Burns and Hull, 1998 and 1999).

In general capsules consist of linear polymers of repeating carbohydrate subunits that might also comprise an amino acid or lipid component. For *E. coli* more than 80 different K-antigens were determined and distinguished into four groups based on biochemical and genetic data (Whitfield and Roberts, 1999). The expression of certain K-antigens (*kps* genes) in ExPEC is strongly associated with particular infections:

The *E. coli* K1 antigen (encoded by the *neu* & *kps* gene clusters) is reported to be essential for intracellular survival and crossing of the blood-brain-barrier and found to be present in most MENEC (Kim, 2002 and 2003). Specifically, the K1 antigen affects *E. coli*-containing vacuoles inside endothelial cells and prevents their fusion with lysosomes (Kim et al., 2003). As such, the K1 antigen plays a crucial role in meningitis. Another study reported on APEC enabled to escape phagocytosis by expression of the K1 antigen, the O78 LPS antigen and P-fimbriae (Mellata et al., 2003). Meanwhile, the K1 antigen is known to avoid serum killing by complement inhibition and is a barrier for the bacteriophage T7 (Wooster et al., 2006; Scholl et al., 2005). However, rather small frequencies of the antigens K1 and K5 in have been reported in UPEC. 1499 *E. coli* isolates from patients with UTIs have been investigated for the presence of K1 antigen and K5 antigen. K1 was detected in 10.5% and K5 in 6.1% of the strains examined (Nimmich et al., 1985). Neither the K1 antigen nor the K5 antigen was detected in any of 273 screened *E. coli* isolates from bovine mastitis (Kaipainen et al., 2002).

Besides antigens K1 and K5, the K2 antigen is known to contribute to ExPEC virulence. A study reported that expression of the K2 capsule by UPEC isolates accounts for the protection against complement-mediated killing and thus affects the pathogenesis of UTIs (Buckles et al., 2009). However, the various functions mediated by the K antigens might be advantageous in mastitis pathogenesis.

Lipopolysaccharides; O-antigens

The lipopolysaccharides (LPS) are the major component of the outer membrane of Gram-negative bacteria comprising three regions (**Figure 4**): (1) the lipid A (endotoxin), which anchors the LPS in the outer membrane, (2) the core oligosaccharide, and (3) the O-antigen or O-polysaccharide (Raetz and Whitfield, 2002; Amor et al., 2000). Lipid A is the most conserved part of LPS and is known to be recognized by the CD14/TLR4/MD-2 receptor complex on host cells (Raetz and Whitfield, 2002; Kawai and Akira, 2010). The O-antigenic polysaccharide (OPS) is highly polymorphic and specific for each serotype. For *E. coli*, more than 180 different O-antigens have been described (Stenutz et al., 2006). They are considered advantageous in colonizing specific niches and essential for the full

function and virulence of bacteria (Moran et al., 2009). Bacteria modify their O-antigens by adding positively charged moieties that prevent the electrostatic interaction of defensins with bacterial surfaces (Thomassin et al., 2012). Moreover, the surface-displayed LPS accounts for many virulence-associated properties: Such as resistance to detergents, hydrophobic antibiotics or organic acids (Barua et al., 2002), adherence to eukaryotic cells (Jacques, 1996; Cohen et al., 1985) and serum resistance due to complement inhibition (Reeves, 1995). Especially the latter is believed to be dependent on the O-polysaccharide chain length (Reeves, 1995; Porat et al., 1992).

Recent studies on the O-antigenic polysaccharide of the bovine mastitis isolate *E. coli* serotype O174 suggest different subtypes of the O174 O-antigen. This supports the idea of evolutionary pressure due to host-pathogen interactions (Duda et al., 2013).

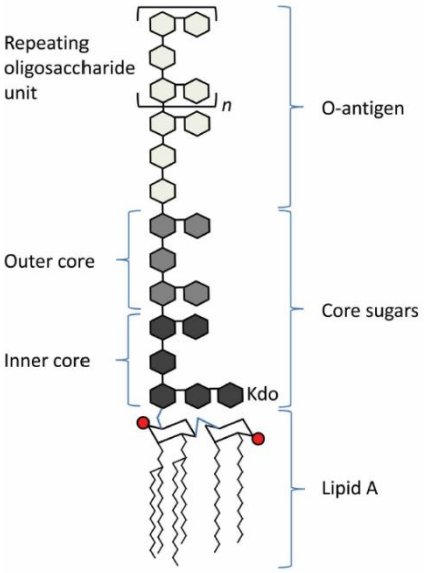

Figure 4: Structural organization of LPS (from Maeshima and Fernandez, 2013). The three regions of LPS from the bottom: lipid A (chair structure indicates di-glucosamine head group, red circles indicate phosphate groups, squiggly lines indicate acyl chains), core sugars and O-antigen, which consists of repeating units (denoted in brackets with an “n”) of oligosaccharides.

For bovine neutrophils it has been reported that binding of LPS to membrane-bound CD14 causes release of TNFα and sepsis (Paape et al., 2003). It is common knowledge that Lipid A as component of LPS acts as prototypical endotoxin, because it binds to the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in many cell types, but especially in macrophages and B cells (Raetz and Whitfield, 2002; Kawai and Akira, 2010). This recognition promotes the secretion of pro-inflammatory cytokines in many cell types, but especially in macrophages and B cells (see also section .0.0). The term "LPS challenge" refers to the process of exposing a subject to LPS that may act as a toxin and is a proposed method to elicit the immune

response within in the udder by LPS induction (Schmitz, 2004; Elazar et al., 2010). In contrast to other virulence-associated factors, LPS is supposed to be a key factor of mastitis pathogenesis. It can be anticipated that modulation of the highly polymorphic O-antigen might affect both pathogen recognition and severity of mastitis pathogenesis and may thus differentiate between putative MPEC causing either acute or persistent mastitis.

Outer membrane proteins

Besides the K-antigens and O-antigens mentioned above, certain outer membrane proteins (Omp) are known mediate serum resistance:

Iss is a lipoprotein in the outer membrane and is encoded by *iss* often located on ColV plasmids (Chuba et al., 1989). It has been demonstrated that three alleles of *iss* occur among *E. coli* isolates possibly evolved from a common *bor* precursor of bacteriophage lambda (Johnson et al., 2008a). Iss (Increased serum survival) expression was first associated to confer resistance after the *iss* gene was introduced into an *E. coli* K-12 strain, and consequently the resistance to bactericidal serum was observed to be increased (Binns et al., 1982). It has been supposed that Iss plays a less important role for serum resistance than the K1 capsule due to observations made by exposure of *E. coli* K1 deletion mutants compared to *iss* isogenic mutants (Tivendale et al., 2004). The *iss* gene is found to be highly prevalent in different ExPEC pathogroups. A study reported that 82.7 % of 451 APEC isolates tested were *iss*-positive whereas only a minority (18.3 %) of *E. coli* isolates from avian faeces was tested positive on *iss* (Rodriguez-Siek et al., 2005). The frequency of *iss* in APEC compared to the environmental isolates (avian faeces) lead to the suggestion that strains in possession of *iss* are likely to become extraintestinal pathogenic.

Notably, one of the type 3 *iss* alleles was reported to occur in the genomes of all sequenced ExPEC strains (known at the time of publication of the study) on a prophage element. When the distribution of all the three *iss* alleles was examined among 487 *E. coli* isolates, the *iss* type 3 gene occurred at a high frequency among ExPEC isolates, irrespective of the host source. Moreover, the plasmid-borne *iss* allele (designated type 1) was highly prevalent among APEC and NMEC isolates, but not among UPEC isolates (Johnson et al., 2008a). Studies that investigate the prevalence of *iss,* ignoring host- or source-specific alleles, might lead to confusion when trying to correlate the presence of *iss* to particular a pathotype. Possibly for this reason two studies on *iss* prevalence in *E. coli* isolates from bovine mastitis have inconsistent results: In one study isolates from mastitic cows (n=63) and from different places of cowsheds (n=24 environmental isolates) showed 33 % frequency of *iss* (Blum and Leitner, 2013). In another study the *iss* prevalence was determined to be 16.7 % in the tested *E. coli* isolates (n=144) from bovine mastitis (Suojala et al., 2011). However, both studies claim that Iss was among the three most prevalent VFs found in the *E. coli* isolates from bovine mastitis.

OmpA is a structural protein and probably one of the most prevalent proteins of the outer membrane of *E. coli*. It is important for the outer membrane stability and is composed of two domains: an N-terminal domain anchored within the outer membrane and a C-terminal domain, which is located within the periplasm (Koebnik et al., 2000). It was suggested that OmpA provides physical linkage between the outer membrane and the underlying peptidoglycan layer until a more specific function of OmpA was observed: *E. coli* K1 *ompA*-deletion mutants were significantly more sensitive to sodium dodecyl sulfate (SDS), cholate, acidic environment, high osmolarity, and pooled human serum. Mutations that caused structural changes to the extracellular loops of OmpA did not affect the viability of *E. coli*, while changes at the OmpA beta-barrel, that provides the structural integrity, decreased *E. coli* resistance to environmental stresses (Wang, 2002). The same study showed that *ompA* mutants survived significantly better within brain microvascular endothelial cells than the wild-type strain what anticipates OmpA as major target in mammalian host cell defense. However, OmpA expression was observed by both *E. coli* strains associated with acute and persistent bovine mastitis upon cocultivation with primary bovine mammary gland epithelial cells (Dego et al., 2012). The OmpA expression levels in either acute or persistant isolates were almost equal.

OmpT is another outer membrane protein that provides resistance to defensins by a different mechanism. Particularly defensins can be proteolytically degraded and inactivated by surface or secreted proteases of the outer membrane such as omptins (Thomassin et al., 2012). The *E. coli* K-12 OmpT was reported to efficiently degrade protamine of defensins (Stumpe et al., 1998). Another study reported that the EHEC and EPEC OmpT proteins contribute differently to the degradation of helical AMPs. Only the EHEC OmpT degraded and inactivated AMPs completely to promote bacterial survival, whereas EPEC OmpT poorly degraded the defensins exposed to (Thomassin et al., 2012). Given the fact that on the one hand, EHEC and EPEC are two genetically related bacteria and on the other hand, both EHEC and EPEC showed similar serum resistance statistics within the study, it was suggested that EPEC relies, at least partly, on other mechanisms to resist defensins (Thomassin et al., 2012). Regarding the advantage of providing serum resistance by *E. coli* bovine mastitis isolates we might have a similar situation.

Also **TraT** is supposed to be a surface exclusion lipoprotein and facilitates extracellular protease activity. The TraT lipoprotein is encoded by the *traT* gene carried on conjugative plasmids, such R6-5 or CoIV of *E. coli* (Binns et al., 1982; Agüero et al., 1984). In particular, the resistance to complement is provided by structural and/or functional changes applied the complement proteins and therefore inhibition the interactions between complement bacterial surface (Agüero et al., 1984). TraT has been considered a virulence marker of ExPEC what is supported by the fact that the *traT* gene was

significantly more prevalent in *E. coli* isolates carrying certain antibiotic resistance genes (Lee et al., 2010). In the past, the presence of *traT* (and also *ompA*) was correlated to *E. coli* causing clinical mastitis of sows, in comparison to isolates from faeces, suggesting a role in mastitis pathogenesis (Gerjets et al., 2011). Moreover, in a study on VFs of *E. coli* isolates from bovine clinical mastitis, *traT* was found in 37 % of the Finnish isolates tested (n=160) and 41 % of the Israeli isolates tested (n=113) (Kaipainen et al., 2002). It was therefore the most prevalent virulence factor identified within these two sets of *E. coli* isolates from bovine clinical mastitis.

Iron uptake systems

Iron is essential for almost every living being and used in oxygen transport and storage, DNA synthesis, electron transport chain and peroxide metabolism. Due to the oxic environment, *E. coli* and other bacteria have to take up the merely soluble Fe(III) and then reduce it to Fe(II) which is highly soluble. This makes iron a limiting nutritional factor for survival and growth within the host niche such as body liquids (e.g. mammary secretions) (Chipperfield and Ratledge, 2000). It can also be limited, because of the host response to infection which further reduces the amount of iron available by iron-scavenging proteins such as lactoferrin (Latorre et al., 2010). Lactoferrin binds iron and makes it unavailable to bacteria (Smith and Schanbacher, 1977). In order to utilize the limited iron in the host niche, it has to be acquired by iron uptake systems. One option is to utilize the protein-bound iron complexes of the host such as hemoglobin, transferrin and lactoferrin by expression of specific receptors (Hanson et al., 1992). Another option is the use of siderophores, which compete with host iron-binding proteins.

The **siderophore** gene clusters encoding the enzymes for enterobactin (*ent*) and the ferric di-citrate transport system (*fec*) have a common conserved localization in the *E. coli* core genome and might be found in pathogenic and non-pathogenic *E. coli*. They are therefore considered important fitness traits. Nonetheless, some siderophore gene clusters (e.g. *chu*, *iro*, *iuc*) are specific to genomic locations (e.g. pathogenicity islands) and/or isolates (Luck et al., 2001; Torres and Payne, 1997; Wyckoff et al., 1998; Bäumler et al., 1996; Dobrindt et al., 2003).

Recently, the inactivation of the biosynthetic pathways of several siderophores (enterobactin, salmochelins and yersiniabactin) abolished the virulence of ExPEC in a mouse sepsis model and showed these pathways essential for the survival of ExPEC *in vivo* (Martin et al., 2013). This might be further supported by observations made on phagocytosis and serum susceptibility of *E. coli* bovine mastitis isolates cultured in iron-deplete and iron-replete media. Iron availability during cultivation altered the susceptibility of isolates to phagocytosis by neutrophils, but had no effect on the susceptibility of isolates to the bactericidal activity of serum (Wise et al., 2002). Therefore, iron acquisition of bacteria can be suggested advantageous for survival within milk, especially in presence of lactoferrin, and iron uptake systems might contribute to mastitis pathogenesis.

Toxins

Some *E. coli* pathotypes are associated with the presence and expression of certain toxins (EHEC: Stx1, Stx2, EAST1, EHly1, EHly2; EPEC: EAST1; ETEC: LT-I, LT-II, STa, STb; EAEC: ShET1, Pic, EAST1, Pet; UPEC: Sat, CNF-1, α-Hly). Several toxins are typically expressed by ExPEC:

The cytotoxic necrotizing factor 1 (**CNF-1**) has been determined to activate RhoA, Rac1, and Cdc42 in epithelial cells (Schmidt et al., 1998; Aktories et al., 2000). Rho GTPases are required to maintain the function of tight junctions and thus the intestinal epithelial barrier function. Activation of Rac1 and Cdc42 exerts barrier-stabilizing effects whereas increased stimulation of RhoA/Rho kinase signaling causes intestinal epithelial barrier disruption (Schlegel et al., 2011). Thus, CNF-1 expression is advantageous in immunoevasion of *E. coli*. This might be important during intramammary infections. The CNF-1 gene (*cnf*1), was thought to be highly prevalent within ExPEC and are often associated with a pathogenicity island (Andreu et al., 1997). Specifically, this study reported a CNF-1 distribution of 44-63 % in *E. coli* isolates (total n= 150) from prostatitis, pyelonephritis, cystitis and UTI with predisposing factors. This is in strong contrast to results of a very recent study that reported CNF-1 distribution of 0-3 % in isolates (n= 100) from pyelonephritis and cystitis (Tarchouna et al., 2013). Concerning bovine mastitis, the *cnf*1 prevalence was very low (5 %, n= 20) in *E. coli* isolates from mastitis. This suggests no important role for mastitis pathogenesis (Lipman et al., 1995). The **α-hemolysin** (*hly*) is considered to be an independent predictor of *E. coli* pathogenicity and belongs to the RTX toxin family (Lee et al., 2010). Often located on PAIs or plasmids, the *hly* gene cluster encodes for the toxin biosynthesis and components of its secretion system. The toxin lyzes red blood cells by damaging their cell membrane (Holland et al., 1990).The α-hemolysin is also believed to be widely distributed among ExPEC isolates. α-Hemolysin production was significantly more common in ExPEC isolates from neonatal sepsis and meningitis as compared with faecal isolates (Korhonen et al., 1985). This was further supported by 43-73 % α-hemolysin positive *E. coli* isolates (total n= 150) from prostatitis, pyelonephritis, cystitis and UTI with predisposing factors (Andreu et al., 1997). In contrast, Tarchouna et al. (2013) detected the *hly* determinant in 19 % of the tested UPEC isolates (n= 90). For ExPEC other than UPEC, a study reported 98 % STEC isolates (n= 400) tested positive for the presence of the so-called EHEC hemolysin (Murinda et al., 2004). Among *E. coli* isolates from bovine mastitis (n= 76), 12 % were positive for *hlyA* (Cheng et al., 2012). **Sat**, the secreted autotransporter toxin is a member of the SPATE (serine protease autotransporters of *Enterobacteriaceae*) family. Besides serine protease activity, Sat causes cytopathic effects on various cell types and is predominantly found among UPEC strains (Guyer et al., 2000). Specifically, the active site of Sat is necessary for the protease and cytotoxic activities, contraction of the cytoskeleton, and loss of actin filaments in cultured bladder and kidney cells (Maroncle et al., 2006). Moreover, Sat was reported to degrade specific membrane/cytoskeletal and nucleus-associated proteins. Data on the contribution of Sat to

coliform bovine mastitis are not available so far. Likewise Sat, another ExPEC-associated toxin, the cytolethal distending toxin (**CDT**) could not related to mastitis (Fernandes et al., 2011). Above all, the toxins, which are not specific for ExPEC (CDT 1, EAST1, LT-I, LT-II, STa, STb, Stx1, Stx2, VT1, VT2) occurred in rather low frequencies or were completely absent among the mastitis isolates tested in various studies (Fernandes et al., 2011; Lipman et al., 1995; Ghanbarpour and Oswald, 2010; Suojala et al., 2011; Murinda et al., 2004; Reichardt and Dobrindt, unpublished data). Notably the consistency between the listed studies seems to be highly dependent of the particular origin.

Autotransporter proteins

The toxin Sat is a member of the autotransporter (AT) protein family. AT proteins are surface-exposed or secreted factors (i.e. Sat) that facilitate various virulence-associated functions to Gram-negative pathogens. In *E. coli*, more than 18 different AT proteins have been identified (Zude et al., 2014). All AT proteins share a characteristic structure consisting of three functional domains: (i) an N-terminal signal sequence, which initiates the SecA-dependent transport across the inner membrane into the periplasm, (ii) an α- or passenger domain, which encodes for functional traits, and (iii) an outer membrane embedded C-terminal β- or translocation domain (Desvaux et al., 2004; Benz and Schmidt, 2011). The AT subtype-specific translocation domain appears to be highly homologous, whereas ATs show substantial sequence diversity in their passenger domain that determines their individual functional properties. These various, often multiple functions were reported to contribute to adhesion (Benz and Schmidt, 1989), autoaggregation (Diderichsen, 1980), biofilm formation (Danese et al., 2000), haemagglutination, serum resistance (Henderson and Nataro, 2001), or exhibit protease activity (Pohlner et al., 1987) or toxin activity (Cover, 1996). In former studies these characteristics have been frequently correlated with pathogenesis and therefore, an application as biomarkers for individual extraintestinal pathogenic *E. coli* (ExPEC) or intestinal pathogenic *E. coli* (IPEC) has been proposed. Another study determined the highest prevalence of ATs per strain in phylogroup B2 isolates and showed that AT distribution correlates rather with phylogenetic lineages than with pathotypes. The AT protein UpaI and its positional ortholog EhaC were detected in 93 % of the *E. coli* strains tested (n= 111). Thus, UpaI is the most prevalent AT in *E. coli* irrespective of pathotype or phylogenetic background and was observed to mediate redundant functions in comparison to the ATs characterized within the study (Zude et al., 2014). Specifically UpaI of UPEC strain 536 is contributing to autoaggregation, biofilm formation, and binding to extracellular matrix proteins. The functional redundancy and wide distribution of ATs among pathogenic and non-pathogenic *E. coli* indicates that ATs cannot generally be regarded as specific biomarkers and VFs *per se*. Nevertheless, AT proteins promote colonization of intestinal and extraintestinal sites by ExPEC and thus AT expression could be considered advantageous in mastitis pathogenesis.

II.2.5. What we know about mammary pathogenic *E. coli*

Regardless pathogenic *E. coli* strains can be classified into different pathotypes according to distinct diseases (Kaper et al., 2004), *E. coli* causing mastitis represents itself peculiar. Specific *E. coli* serotypes involved in mastitis could neither be identified by epidemiological studies, nor could a common set of VFs be described for mastitis-causing *E. coli* strains. Furthermore, none of the previously studied phylogenetic groups, VFs or antimicrobial resistance traits were associated with clinical signs, persistence of intramammary infection or clinical recovery from mastitis (Blum and Leitner, 2013; Silva et al., 2013; Cheng et al., 2012; Dogan et al., 2012; Kerro Dego et al., 2012; Fernandes et al., 2011; Suojala et al., 2011; Ghanbarpour and Oswald, 2010; Dyer et al., 2007; Wenz et al., 2006; Bean et al., 2004; Lehtolainen et al., 2003; Kaipainen et al., 2002; Wise et al., 2002; Lipman et al., 1995; Sanchez-Carlo et al., 1984). Instead, it is thought that mastitis-causing *E. coli* are typical commensals (Suojala et al., 2011). However, commensal *E. coli* group mostly into phylogroup A while *E. coli* strains associated to mastitis are wide spread among different phylogenetic groups. Traditionally, the 'cow factors' are held responsible for differences in *E. coli* mastitis severity rather than variations in the bacterial set of VFs or their expression profile (Burvenich et al., 2003). In contrast to the widely accepted opinion that bacterial VFs are not involved in *E. coli* mastitis, large variations have been observed among field cases of *E. coli* mastitis in dairy animals. The severity of the disease can vary from a mild, self-curing to a fatal septic condition. Several field strains (e.g. P4) are highly virulent, and upon experimental infection consistently lead to severe septic mastitis in cows. In contrast, others cause mild mastitis and might result in latency or in chronically infected dairy animals (Döpfer et al., 1999, 2000 and 2001; Dogan et al., 2006). Nevertheless, in analogy to human and avian ExPEC isolates, the high geno- and phenotypic diversity among *E. coli* mastitis isolates may contribute to the variation in aetiopathology. Thus, mastitis-causing *E. coli* might make use of the same strategies used by other ExPEC including adhesion to and invasion into epithelial cells, immune evasion, replication, and tissue damage characterized by necrosis and apoptosis of epithelial cells. It also has been discussed that bacterial VFs may contribute to the disease (Shpigel et al., 2008). However, the different strains carry different sets of virulence genes and the accumulation of such traits may increase their virulence potential.

II.3. Aims of this study

Commensal and pathogenic *E. coli* variants adapt their gene expression contributing to survival and colonization during the transition between the environment and host. In the past, specific gene subsets and traits have been correlated with an increased potential of *E. coli* strains to either cause intestinal or extraintestinal diseases in humans and many animal hosts (Leimbach et al., 2013). However, to date a marked prevalence of many known virulence-associated genes of ExPEC and IPEC

among *E. coli* mastitis isolates has not been published. This indicates that the *E. coli* factors and traits required for the development of the variant forms of mastitis still remain unexplored or at least unidentified.

This project aimed at the identification and characterization of virulence-associated traits from *E. coli* strains associated to bovine mastitis. Specifically, the *E. coli* model mastitis isolates 1303 (acute mastitis isolate) and ECC-1470 (persistent mastitis isolate) should be comprehensively examined. The main aim of this study was to study the distribution and regulation of virulence-associated factors in these isolates in order to better understand the molecular basis of pathogenesis during *E. coli* mastitis. For this purpose, transcriptomic analyses of the two mastitis isolates performed under different growth conditions should allow to screen for candidate genes involved in bacterium-host interaction, fitness or even pathogenicity during bovine mastitis. Specifically, the virulence-associated gene content of the *E. coli* mastitis isolates 1303 and ECC-1470 should be assessed based on BLAST analyses of their draft genome sequences. A collection of *E. coli* isolates from bovine mastitis or from bovine faeces should be screened by PCR for the prevalence of selected autotransporter determinants.

In order to study fitness traits and gene expression of *E. coli* mastitis isolates suitable *in vitro* test assays should be established, which mimic the environment and growth conditions in the bovine mammary gland *in vivo*. Fitness traits of selected bovine mastitis and/or faecal *E. coli* strains should be identified and analyzed in comparative phenotypic assays, i.e. the viability and growth under different conditions mimicking the environment during initial pathogenesis should be investigated.

The transcriptional response of the mastitis isolates 1303 and ECC-1470 should be comprehensively assessed under growth conditions mimicking the initial stages of bovine mastitis pathogenesis by *in vitro* transcriptome analysis in order to identify (i) candidate genes involved in bacterium-host interaction upon cocultivation with MAC-T immortalized bovine mammary gland epithelial cells, (ii) candidate genes that enable mastitis isolates to quickly adapt to and survive the antimicrobial conditions within bovine native milk whey.

As a prerequisite to study bacterial gene expression upon growth under *in vivo*-like conditions, i.e. in raw milk whey, protocols for the enrichment of bacterial cells from milk whey and for the purification of bacterial RNA from milk whey should be established.

These data should allow us new insights into bacterial traits, which contribute to the adaptation of *E. coli* to host conditions in the bovine mammary gland and thus, may also contribute to mastitis.

III. MATERIAL

III.1. STRAINS

All bacterial strains used in this study are listed in **Table 6**.

Table 6: Bacterial strains used in this study.

Strain	Source/origin	Serotype	Reference
E. coli 1303	acute bovine mastitis		(Petzl et al., 2008)
ECC-1470	persistant bovine mastitis		(Dogan et al., 2006)
UPEC 536	clinical pyelonephritis	O6:K15:H31	(Berger et al., 1982)
MG1655	faeces	K-12	(IMIB strain collection, Würzburg)
RZ422	clinical pyelonephritis	O6:K14:H-	(IMIB strain collection, Würzburg)
RZ451	clinical pyelonephritis	O6:K+:H31	(IMIB strain collection, Würzburg)
RZ479	clinical pyelonephritis	O6:K+:H-	(IMIB strain collection, Würzburg)
RZ505	clinical pyelonephritis	O6:K14:H-	(IMIB strain collection, Würzburg)
RZ532	clinical pyelonephritis	O6:K-:H31	(IMIB strain collection, Würzburg)
HK8	sepsis	-	(IMIB strain collection, Würzburg)
HK24	sepsis	-	(IMIB strain collection, Würzburg)
RZ454	clinical pyelonephritis	O6:K2:H-	(IMIB strain collection, Würzburg)
20A1	faeces	-	(IMIB strain collection, Würzburg)
20A1U	faeces	-	(IMIB strain collection, Würzburg)
22A2	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
22B2U	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
J96	clinical pyelonephritis	O4:K:H5	(IMIB strain collection, Würzburg)
J96-M1	clinical pyelonephritis	O4:K:H5	(IMIB strain collection, Würzburg)
AD110	clinical pyelonephritis	O4:K6	(IMIB strain collection, Würzburg)
AC/I	sepsis (avian)	O78	(IMIB strain collection, Würzburg)
2980	clinical pyelonephritis	O18ac:K5	(IMIB strain collection, Würzburg)
E642	sepsis	-	(IMIB strain collection, Würzburg)
CFT073	clinical pyelonephritis	O6:K2:H1	(IMIB strain collection, Würzburg)
764	faeces	O18:K5:H5/11	(IMIB strain collection, Würzburg)
764-2	faeces	O18:K5:H5/11	(IMIB strain collection, Würzburg)
E-B35	clinical pyelonephritis	O4:K12:H5	(IMIB strain collection, Würzburg)
7521/94-1	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
RZ-436	clinical pyelonephritis	O6:K13:H1	(IMIB strain collection, Würzburg)
RZ-439	clinical pyelonephritis	O6:K5:H1	(IMIB strain collection, Würzburg)
RZ-441	clinical pyelonephritis	O6:K5:H1	(IMIB strain collection, Würzburg)
RZ-443	clinical pyelonephritis	O6:K5:H-	(IMIB strain collection, Würzburg)
RZ-446	clinical pyelonephritis	O6:K53:H1	(IMIB strain collection, Würzburg)
RZ-458	clinical pyelonephritis	O6:K2:H1	(IMIB strain collection, Würzburg)
RZ-468	clinical pyelonephritis	O6:K5:H1	(IMIB strain collection, Würzburg)
RZ-475	clinical pyelonephritis	O6:K5:H1	(IMIB strain collection, Würzburg)
RZ-495	clinical pyelonephritis	O6:K5:H-	(IMIB strain collection, Würzburg)
RZ-500	clinical pyelonephritis	O6:K5:H1	(IMIB strain collection, Würzburg)
RZ-525	clinical pyelonephritis	O6:K5:H1	(IMIB strain collection, Würzburg)
RZ-526	clinical pyelonephritis	O6:K5:H1	(IMIB strain collection, Würzburg)
ECOR 51	faeces	O25:Hnt	(IMIB strain collection, Würzburg)
ECOR 52	faeces	O25:H1	(IMIB strain collection, Würzburg)
ECOR 53	faeces	O4:H-	(IMIB strain collection, Würzburg)

Strain	Source/origin	Serotype	Reference
ECOR 54	faeces	O25:H1	(IMIB strain collection, Würzburg)
ECOR 57	faeces	Ont:Hnt	(IMIB strain collection, Würzburg)
ECOR 58	faeces	O112:H8	(IMIB strain collection, Würzburg)
ECOR 60	clinical pyelonephritis	O4:Hnt	(IMIB strain collection, Würzburg)
ECOR 63	faeces	Ont:Hnt	(IMIB strain collection, Würzburg)
ECOR 64	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
ECOR 65	faeces	Ont:H10	(IMIB strain collection, Würzburg)
2E2U	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
3D5	faeces	-	(IMIB strain collection, Würzburg)
13A1	faeces	-	(IMIB strain collection, Würzburg)
HK54	sepsis	-	(IMIB strain collection, Würzburg)
BK658	neonatal bacterial meningitis	O75:K1:H7	(IMIB strain collection, Würzburg)
1G1U	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
1H1	faeces	-	(IMIB strain collection, Würzburg)
1H1U	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
19A1	faeces	-	(IMIB strain collection, Würzburg)
1G1	faeces	-	(IMIB strain collection, Würzburg)
ECOR 66	faeces	O4:H40	(IMIB strain collection, Würzburg)
IHE3034	neonatal bacterial meningitis	O18:K1:H7/9	(IMIB strain collection, Würzburg)
IHE3036	neonatal bacterial meningitis	O18:H7:K1	(IMIB strain collection, Würzburg)
IHE3080	neonatal bacterial meningitis	O18:H7:K1	(IMIB strain collection, Würzburg)
RS218	neonatal bacterial meningitis	O18ac:H7:K1	(IMIB strain collection, Würzburg)
RS226	neonatal bacterial meningitis	O18ac:H7:K1	(IMIB strain collection, Würzburg)
B13155	neonatal bacterial meningitis	-	(IMIB strain collection, Würzburg)
B616	sepsis	-	(IMIB strain collection, Würzburg)
2E1U	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
16A2U	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
HK2	sepsis	-	(IMIB strain collection, Würzburg)
W1825	sepsis	-	(IMIB strain collection, Würzburg)
4405/1	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
S5	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
HK1	sepsis	-	(IMIB strain collection, Würzburg)
HK58	sepsis	-	(IMIB strain collection, Würzburg)
ECOR 7	-	-	(IMIB strain collection, Würzburg)
ECOR 62	clinical pyelonephritis	O2:H-	(IMIB strain collection, Würzburg)
8B1	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
Ve1140	neonatal bacterial meningitis	-	(IMIB strain collection, Würzburg)
B10363	neonatal bacterial meningitis blood cult	-	(IMIB strain collection, Würzburg)
E351	sepsis, blood culture	-	(IMIB strain collection, Würzburg)
F18	faeces	-	(IMIB strain collection, Würzburg)
F18Col -	faeces	-	(IMIB strain collection, Würzburg)
ECOR 50	clinical pyelonephritis	O2:H-	(IMIB strain collection, Würzburg)
RS176	neonatal bacterial meningitis	O7:K1	(IMIB strain collection, Würzburg)
Ve239	neonatal bacterial meningitis	-	(IMIB strain collection, Würzburg)
ECOR 61	faeces	O2:H-	(IMIB strain collection, Würzburg)
RZ411	clinical pyelonephritis	O6:K-:H1	(IMIB strain collection, Würzburg)
E457	sepsis	-	(IMIB strain collection, Würzburg)
1E2	faeces	-	(IMIB strain collection, Würzburg)
AE5	faeces	-	(IMIB strain collection, Würzburg)

Strain	Source/origin	Serotype	Reference
2A1	faeces	-	(IMIB strain collection, Würzburg)
2A2	faeces	-	(IMIB strain collection, Würzburg)
3B5	faeces	-	(IMIB strain collection, Würzburg)
3N1	faeces	-	(IMIB strain collection, Würzburg)
3N2	faeces	-	(IMIB strain collection, Würzburg)
3N5	faeces	-	(IMIB strain collection, Würzburg)
5A1	faeces	-	(IMIB strain collection, Würzburg)
8B2	faeces	-	(IMIB strain collection, Würzburg)
16A3	faeces	-	(IMIB strain collection, Würzburg)
16B1	faeces	-	(IMIB strain collection, Würzburg)
A284	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
RB9	Sheep pathogen	-	(IMIB strain collection, Würzburg)
HK4	sepsis	-	(IMIB strain collection, Würzburg)
HK17	sepsis	-	(IMIB strain collection, Würzburg)
HK19	sepsis	-	(IMIB strain collection, Würzburg)
ECOR 1	faeces	Ont:Hnt	(IMIB strain collection, Würzburg)
ECOR 23	faeces	O86:H43	(IMIB strain collection, Würzburg)
ECOR 25	faeces	Ont:Hnt	(IMIB strain collection, Würzburg)
ECOR 28	faeces	O104:H-	(IMIB strain collection, Würzburg)
ECOR 31	faeces	O79:H43	(IMIB strain collection, Würzburg)
ECOR 32	faeces	O7:H21	(IMIB strain collection, Würzburg)
ECOR 40	clinical pyelonephritis	O7:H-	(IMIB strain collection, Würzburg)
ECOR 42	faeces	Ont:H26	(IMIB strain collection, Würzburg)
ECOR 48	clinical pyelonephritis	-	(IMIB strain collection, Würzburg)
A12	-	-	(IMIB strain collection, Würzburg)
ECOR 56	faeces	O6:H1	(IMIB strain collection, Würzburg)
ECOR 71	clinical pyelonephritis	O78:H-	(IMIB strain collection, Würzburg)
EDL1284	EIEC	O124:H-	(IMIB strain collection, Würzburg)
76-5	EIEC	O143	(IMIB strain collection, Würzburg)
E2348/69	EPEC	O127:H6	(IMIB strain collection, Würzburg)
179/2	EPEC	O55:H6	(IMIB strain collection, Würzburg)
156A	EPEC	O55:H6	(IMIB strain collection, Würzburg)
37-4	EPEC	O55:H-	(IMIB strain collection, Würzburg)
933W	EHEC	O157:H7	(IMIB strain collection, Würzburg)
86-24	EHEC	O157:H7	(IMIB strain collection, Würzburg)
SF493/89	EHEC	O157:H-	(IMIB strain collection, Würzburg)
3574/92	EHEC	O157:H7	(IMIB strain collection, Würzburg)
2907/97	EHEC	-	(IMIB strain collection, Würzburg)
5720/96	EHEC	O26:H11	(IMIB strain collection, Würzburg)
3697/97	EHEC	-	(IMIB strain collection, Würzburg)
ED142	EHEC	O111	(IMIB strain collection, Würzburg)
PIG E57	EHEC	-	(IMIB strain collection, Würzburg)
Feb 45	EHEC	-	(IMIB strain collection, Würzburg)
EDL880	EHEC	-	(IMIB strain collection, Würzburg)
5714/96	EHEC	-	(IMIB strain collection, Würzburg)
G1253	ETEC	-	(IMIB strain collection, Würzburg)
147/1	ETEC	O128:H-	(IMIB strain collection, Würzburg)
284/97	ETEC	-	(IMIB strain collection, Würzburg)
297/87	ETEC	-	(IMIB strain collection, Würzburg)

Strain	Source/origin	Serotype	Reference
164/82	ETEC	-	(IMIB strain collection, Würzburg)
117/86	ETEC	-	(IMIB strain collection, Würzburg)
6061/97	EHEC	O26	(IMIB strain collection, Würzburg)
5080/97	EHEC	O26	(IMIB strain collection, Würzburg)
4104/97	EHEC	O26	(IMIB strain collection, Würzburg)
8574/96	EHEC	O26	(IMIB strain collection, Würzburg)
1639/77	EHEC	O111:H-	(IMIB strain collection, Würzburg)
E1392-75	ETEC	O6:H16	(IMIB strain collection, Würzburg)
278485-2	ETEC	-	(IMIB strain collection, Würzburg)
VM75688	ETEC	-	(IMIB strain collection, Würzburg)
350 C1A	ETEC	-	(IMIB strain collection, Würzburg)
7476 A	ETEC	-	(IMIB strain collection, Würzburg)
E 34420 A	ETEC	-	(IMIB strain collection, Würzburg)
Feb 45	EHEC	-	(IMIB strain collection, Würzburg)
04259-01	EHEC	-	(IMIB strain collection, Würzburg)
04037-01	EHEC	-	(IMIB strain collection, Würzburg)
09282-01	EHEC	-	(IMIB strain collection, Würzburg)
01594-01	EHEC	-	(IMIB strain collection, Würzburg)
4738/96	EHEC	O113	(IMIB strain collection, Würzburg)
2851/96	EHEC	O103	(IMIB strain collection, Würzburg)
3937/97	EHEC	O62:H-	(IMIB strain collection, Würzburg)
4789/97	EHEC	O96:H-	(IMIB strain collection, Würzburg)
3115/97	EHEC	O128:H2	(IMIB strain collection, Würzburg)
3172/97	EHEC	O128:H2	(IMIB strain collection, Würzburg)
2455/99	EHEC	O128:H2	(IMIB strain collection, Würzburg)
3117/98	EHEC	O128:H-	(IMIB strain collection, Würzburg)
4736/98	EHEC	O128:H	(IMIB strain collection, Würzburg)
0653/99	EHEC	O128:Hnt	(IMIB strain collection, Würzburg)
4941/97	EHEC	Ont:H- (O26)	(IMIB strain collection, Würzburg)
3615/99	EHEC	O8:H10	(IMIB strain collection, Würzburg)
E25/02	EHEC	Ont:H19	(IMIB strain collection, Würzburg)
E57	EHEC	O138	(IMIB strain collection, Würzburg)
24059/97	EHEC	Ont:H10	(IMIB strain collection, Würzburg)
3229/98	EHEC	O8:H-	(IMIB strain collection, Würzburg)
	EIEC	O164	(IMIB strain collection, Würzburg)
5157/96	EHEC	O26:H11	(IMIB strain collection, Würzburg)
4356/96	EHEC	O26:H-	(IMIB strain collection, Würzburg)
1530/99	EHEC	O26:H11	(IMIB strain collection, Würzburg)
1676/99	EHEC	O26:H11	(IMIB strain collection, Würzburg)
6416/87	EHEC	O26:H-	(IMIB strain collection, Würzburg)
2514/99	EHEC	O26:H-	(IMIB strain collection, Würzburg)
1226/65	EHEC	O26:H11	(IMIB strain collection, Würzburg)
4791/97	EHEC	O111:H-	(IMIB strain collection, Würzburg)
4794/97	EHEC	O111:H-	(IMIB strain collection, Würzburg)
4141/96	EHEC	O111	(IMIB strain collection, Würzburg)
E 20738 A	ETEC	-	(IMIB strain collection, Würzburg)
312/00	EPEC	Ont:Hnt	(IMIB strain collection, Würzburg)
540/00	chronic bovine mastitis	O154:H4	(IMIB strain collection, Würzburg)
ECA-5019	chronic bovine mastitis	-	(Schukken, Y., USA)

Strain	Source/origin	Serotype	Reference
ECA-O157	chronic bovine mastitis	-	(Dogan et al., 2006)
ECC-Z	chronic bovine mastitis	-	(Dogan et al., 2006)
ECA-727	chronic bovine mastitis	-	(Dogan et al., 2006)
ECA-5641	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-5549	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-4789	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-B	chronic bovine mastitis	-	(Dogan et al., 2006)
ECA-5614	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-3722	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-4365	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-4302	chronic bovine mastitis	-	(Schukken, Y., USA)
ECC-M	chronic bovine mastitis	-	(Dogan et al., 2006)
ECA-3471	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-5579	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-5406	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-4707	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-5362	chronic bovine mastitis	-	(Schukken, Y., USA)
ECA-5523	chronic bovine mastitis	-	(Schukken, Y., USA)
800/12d	chronic bovine mastitis	-	(Schukken, Y., USA)
5366a	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
5550d	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
629/8b	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
905/67B	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
1223/A	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
3290/C	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
3290/D	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
1223/6C	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
3222A	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
3222A	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
2940C	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
2772a	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
3242/A	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
3238/B+D	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
1200/47C	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
2906 d	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
1111/18a	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
1099/3a	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
2940/C	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
1196/158a	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
1173/49A	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
1139/6A	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
118/12C	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
0001/08	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
725/07	clinical bovine mastitis+sepsis)	-	(Tiergesundheitsdienst Bayern)
362/06 rot	clinical bovine mastitis	-	(Tiergesundheitsdienst Bayern)
441/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
390/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
000/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
278/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)

Strain	Source/origin	Serotype	Reference
UVM2	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
588/06	clinical bovine mastitis	-	(Wellnitz, O., Bern)
583/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
500/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
606/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
465/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
477/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
461/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
470/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
22/2007	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
75/2007	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
95/06	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
131/2007	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
191/2007	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
146/2007	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-3793	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-4003	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-3969	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-4080	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-4097	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-0307-778	chronic bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-0314-305	subclinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-0509-284	subclinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
07-0314-727	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
332/07	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
263/07	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
302/07	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
294/07	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
333/07	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
297/07	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
300/07	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
5234	clinical bovine mastitis	-	(Petzel, W., Oberschleißheim)
5232	clinical Mastitis	-	(Wieler, L., Berlin)
5228	clinical Mastitis	-	(Wieler, L., Berlin)
5229	clinical Mastitis	-	(Wieler, L., Berlin)
5250	clinical Mastitis	-	(Wieler, L., Berlin)
5251	clinical Mastitis	-	(Wieler, L., Berlin)
5249	clinical Mastitis	-	(Wieler, L., Berlin)
5248	clinical Mastitis	-	(Wieler, L., Berlin)
5243	clinical Mastitis	-	(Wieler, L., Berlin)
5252	clinical Mastitis	-	(Wieler, L., Berlin)
5244	clinical Mastitis	-	(Wieler, L., Berlin)
5246	clinical Mastitis	-	(Wieler, L., Berlin)
5245	clinical Mastitis	-	(Wieler, L., Berlin)
5247	clinical Mastitis	-	(Wieler, L., Berlin)
5225	clinical Mastitis	-	(Wieler, L., Berlin)
5223	clinical Mastitis	-	(Wieler, L., Berlin)
5242	clinical Mastitis	-	(Wieler, L., Berlin)
5241	clinical Mastitis	-	(Wieler, L., Berlin)

Strain	Source/origin	Serotype	Reference
5237	clinical Mastitis	-	(Wieler, L., Berlin)
5241	clinical Mastitis	-	(Wieler, L., Berlin)
5224	clinical Mastitis	-	(Wieler, L., Berlin)
5226	clinical Mastitis	-	(Wieler, L., Berlin)
5239	clinical Mastitis	-	(Wieler, L., Berlin)
5235	clinical Mastitis	-	(Wieler, L., Berlin)
5236	clinical Mastitis	-	(Wieler, L., Berlin)
5238	clinical Mastitis	-	(Wieler, L., Berlin)
5227	clinical Mastitis	-	(Wieler, L., Berlin)
5231	clinical Mastitis	-	(Wieler, L., Berlin)
5233	clinical Mastitis	-	(Wieler, L., Berlin)
5238	clinical Mastitis	-	(Wieler, L., Berlin)
32	clinical Mastitis	-	(Wieler, L., Berlin)
81	clinical bovine mastitis	-	(Wieler, L., Berlin)
130	clinical bovine mastitis	-	(Wieler, L., Berlin)
131	clinical bovine mastitis	-	(Wieler, L., Berlin)
132	clinical bovine mastitis	-	(Wieler, L., Berlin)
133	clinical bovine mastitis	-	(Wieler, L., Berlin)
134	clinical bovine mastitis	-	(Wieler, L., Berlin)
135	clinical bovine mastitis	-	(Wieler, L., Berlin)
136	clinical bovine mastitis	-	(Wieler, L., Berlin)
137	clinical bovine mastitis	-	(Wieler, L., Berlin)
180	clinical bovine mastitis	-	(Wieler, L., Berlin)
184	clinical bovine mastitis	-	(Wieler, L., Berlin)
186	clinical bovine mastitis	-	(Wieler, L., Berlin)
189	clinical bovine mastitis	-	(Wieler, L., Berlin)
191	clinical bovine mastitis	-	(Wieler, L., Berlin)
193	clinical bovine mastitis	-	(Wieler, L., Berlin)
201	clinical bovine mastitis	-	(Wieler, L., Berlin)
204	clinical bovine mastitis	-	(Wieler, L., Berlin)
212	clinical bovine mastitis	-	(Wieler, L., Berlin)
213	clinical bovine mastitis	-	(Wieler, L., Berlin)
222	clinical bovine mastitis	-	(Wieler, L., Berlin)
224	clinical bovine mastitis	-	(Wieler, L., Berlin)
226	clinical bovine mastitis	-	(Wieler, L., Berlin)
856	clinical bovine mastitis	-	(Wieler, L., Berlin)
1070	clinical bovine mastitis	-	(Wieler, L., Berlin)
1080	clinical bovine mastitis	-	(Wieler, L., Berlin)
1102	clinical bovine mastitis	-	(Wieler, L., Berlin)
1109	clinical bovine mastitis	-	(Wieler, L., Berlin)
1111	clinical bovine mastitis	-	(Wieler, L., Berlin)
1115	clinical bovine mastitis	-	(Wieler, L., Berlin)
1116	clinical bovine mastitis	-	(Wieler, L., Berlin)
1121	clinical bovine mastitis	-	(Wieler, L., Berlin)
1122	clinical bovine mastitis	-	(Wieler, L., Berlin)
1136	clinical bovine mastitis	-	(Wieler, L., Berlin)
1176	clinical bovine mastitis	-	(Wieler, L., Berlin)
1192	clinical bovine mastitis	-	(Wieler, L., Berlin)
1366	clinical bovine mastitis	-	(Wieler, L., Berlin)

Strain	Source/origin	Serotype	Reference
1367	clinical bovine mastitis	-	(Wieler, L., Berlin)
1436	clinical bovine mastitis	-	(Wieler, L., Berlin)
1472	clinical bovine mastitis	-	(Wieler, L., Berlin)
1476	clinical bovine mastitis	-	(Wieler, L., Berlin)
1477	clinical bovine mastitis	-	(Wieler, L., Berlin)
1486	clinical bovine mastitis	-	(Wieler, L., Berlin)
1487	clinical bovine mastitis	-	(Wieler, L., Berlin)
1493	clinical bovine mastitis	-	(Wieler, L., Berlin)
1501	clinical bovine mastitis	-	(Wieler, L., Berlin)
1503	clinical bovine mastitis	-	(Wieler, L., Berlin)
1504	clinical bovine mastitis	-	(Wieler, L., Berlin)
2567	clinical bovine mastitis	-	(Wieler, L., Berlin)
2585	clinical bovine mastitis	-	(Wieler, L., Berlin)
2606	clinical bovine mastitis	-	(Wieler, L., Berlin)
2607	clinical bovine mastitis	-	(Wieler, L., Berlin)
2608	clinical bovine mastitis	-	(Wieler, L., Berlin)
2609	clinical bovine mastitis	-	(Wieler, L., Berlin)
2805	clinical bovine mastitis	-	(Wieler, L., Berlin)
2869	clinical bovine mastitis	-	(Wieler, L., Berlin)
2873	clinical bovine mastitis	-	(Wieler, L., Berlin)
2875	clinical bovine mastitis	-	(Wieler, L., Berlin)
2882	clinical bovine mastitis	-	(Wieler, L., Berlin)
2907	clinical bovine mastitis	-	(Wieler, L., Berlin)
2910	clinical bovine mastitis	-	(Wieler, L., Berlin)
2913	clinical bovine mastitis	-	(Wieler, L., Berlin)
2914	clinical bovine mastitis	-	(Wieler, L., Berlin)
2917	clinical bovine mastitis	-	(Wieler, L., Berlin)
2919	clinical bovine mastitis	-	(Wieler, L., Berlin)
2922	clinical bovine mastitis	-	(Wieler, L., Berlin)
2924	clinical bovine mastitis	-	(Wieler, L., Berlin)
2925	clinical bovine mastitis	-	(Wieler, L., Berlin)
3243	clinical bovine mastitis	-	(Wieler, L., Berlin)
3352	clinical bovine mastitis	-	(Wieler, L., Berlin)
3884	clinical bovine mastitis	-	(Wieler, L., Berlin)
3887	clinical bovine mastitis	-	(Wieler, L., Berlin)
3888	clinical bovine mastitis	-	(Wieler, L., Berlin)
4616	clinical bovine mastitis	-	(Wieler, L., Berlin)
4618	clinical bovine mastitis	-	(Wieler, L., Berlin)
4631	clinical bovine mastitis	-	(Wieler, L., Berlin)
4833	clinical bovine mastitis	-	(Wieler, L., Berlin)
4841	clinical bovine mastitis	-	(Wieler, L., Berlin)
4908	clinical bovine mastitis	-	(Wieler, L., Berlin)
5120	clinical bovine mastitis	-	(Wieler, L., Berlin)
5121	clinical bovine mastitis	-	(Wieler, L., Berlin)
5289	clinical bovine mastitis	-	(Wieler, L., Berlin)
5330	clinical bovine mastitis	-	(Wieler, L., Berlin)
5335	clinical bovine mastitis	-	(Wieler, L., Berlin)
5337	clinical bovine mastitis	-	(Wieler, L., Berlin)
5364	clinical bovine mastitis	-	(Wieler, L., Berlin)

Strain	Source/origin	Serotype	Reference
5386	clinical bovine mastitis	-	(Wieler, L., Berlin)
5415	clinical bovine mastitis	-	(Wieler, L., Berlin)
5420	clinical bovine mastitis	-	(Wieler, L., Berlin)
5430	clinical bovine mastitis	-	(Wieler, L., Berlin)
5432	clinical bovine mastitis	-	(Wieler, L., Berlin)
5436	clinical bovine mastitis	-	(Wieler, L., Berlin)
6404	bovine faeces	-	(Wieler, L., Berlin)
6433	bovine faeces	-	(Wieler, L., Berlin)
2275	bovine faeces	-	(Petzel, W., Oberschleißheim)
2276	bovine faeces	-	(Petzel, W., Oberschleißheim)
2278	bovine faeces	-	(Petzel, W., Oberschleißheim)
2279	bovine faeces	-	(Petzel, W., Oberschleißheim)
2280	bovine faeces	-	(Petzel, W., Oberschleißheim)
2281	bovine faeces	-	(Petzel, W., Oberschleißheim)
2282	bovine faeces	-	(Petzel, W., Oberschleißheim)
2283	bovine faeces	-	(Petzel, W., Oberschleißheim)
2284	bovine faeces	-	(Petzel, W., Oberschleißheim)
2285	bovine faeces	-	(Petzel, W., Oberschleißheim)
2286	bovine faeces	-	(Petzel, W., Oberschleißheim)
2287	bovine faeces	-	(Petzel, W., Oberschleißheim)
2288a	bovine faeces	-	(Petzel, W., Oberschleißheim)
2288b	bovine faeces	-	(Petzel, W., Oberschleißheim)
2289	bovine faeces	-	(Petzel, W., Oberschleißheim)
2292	bovine faeces	-	(Petzel, W., Oberschleißheim)
2295	bovine faeces	-	(Petzel, W., Oberschleißheim)
2298	bovine faeces	-	(Petzel, W., Oberschleißheim)
2299	bovine faeces	-	(Petzel, W., Oberschleißheim)
2300	bovine faeces	-	(Petzel, W., Oberschleißheim)
2305	bovine faeces	-	(Petzel, W., Oberschleißheim)
2306	bovine faeces	-	(Petzel, W., Oberschleißheim)
2307	bovine faeces	-	(Petzel, W., Oberschleißheim)
2308	bovine faeces	-	(Petzel, W., Oberschleißheim)
2309	bovine faeces	-	(Petzel, W., Oberschleißheim)
2310	bovine faeces	-	(Petzel, W., Oberschleißheim)
2311	bovine faeces	-	(Petzel, W., Oberschleißheim)
2312	bovine faeces	-	(Petzel, W., Oberschleißheim)
2315	bovine faeces	-	(Petzel, W., Oberschleißheim)
2318	bovine faeces	-	(Petzel, W., Oberschleißheim)
2319	bovine faeces	-	(Petzel, W., Oberschleißheim)
2320	bovine faeces	-	(Petzel, W., Oberschleißheim)
2322	bovine faeces	-	(Petzel, W., Oberschleißheim)
2323	bovine faeces	-	(Petzel, W., Oberschleißheim)
2325	bovine faeces	-	(Petzel, W., Oberschleißheim)
2326	bovine faeces	-	(Petzel, W., Oberschleißheim)
2327	bovine faeces	-	(Petzel, W., Oberschleißheim)
2328	bovine faeces	-	(Petzel, W., Oberschleißheim)
2329	bovine faeces	-	(Petzel, W., Oberschleißheim)
2330	bovine faeces	-	(Petzel, W., Oberschleißheim)
2331	bovine faeces	-	(Petzel, W., Oberschleißheim)

Strain	Source/origin	Serotype	Reference
2332	bovine faeces	-	(Petzel, W., Oberschleißheim)
2333	bovine faeces	-	(Petzel, W., Oberschleißheim)
2335	bovine faeces	-	(Petzel, W., Oberschleißheim)
2338	bovine faeces	-	(Petzel, W., Oberschleißheim)
2339	bovine faeces	-	(Petzel, W., Oberschleißheim)
2340	bovine faeces	-	(Petzel, W., Oberschleißheim)
2341	bovine faeces	-	(Petzel, W., Oberschleißheim)
2342	bovine faeces	-	(Petzel, W., Oberschleißheim)
2343	bovine faeces	-	(Petzel, W., Oberschleißheim)
2344	bovine faeces	-	(Petzel, W., Oberschleißheim)
2345	bovine faeces	-	(Petzel, W., Oberschleißheim)
2346	bovine faeces	-	(Petzel, W., Oberschleißheim)
2350	bovine faeces	-	(Petzel, W., Oberschleißheim)
2351	bovine faeces	-	(Petzel, W., Oberschleißheim)
2352	bovine faeces	-	(Petzel, W., Oberschleißheim)
2353	bovine faeces	-	(Petzel, W., Oberschleißheim)
2354	bovine faeces	-	(Petzel, W., Oberschleißheim)
2355	bovine faeces	-	(Petzel, W., Oberschleißheim)
2356	bovine faeces	-	(Petzel, W., Oberschleißheim)
2357	bovine faeces	-	(Petzel, W., Oberschleißheim)
2358	bovine faeces	-	(Petzel, W., Oberschleißheim)
2359	bovine faeces	-	(Petzel, W., Oberschleißheim)
2360	bovine faeces	-	(Petzel, W., Oberschleißheim)
2363	bovine faeces	-	(Petzel, W., Oberschleißheim)
2364	bovine faeces	-	(Petzel, W., Oberschleißheim)
2365	bovine faeces	-	(Petzel, W., Oberschleißheim)
2366	bovine faeces	-	(Petzel, W., Oberschleißheim)
2367	bovine faeces	-	(Petzel, W., Oberschleißheim)
2370	bovine faeces	-	(Petzel, W., Oberschleißheim)
2371	bovine faeces	-	(Petzel, W., Oberschleißheim)
2372	bovine faeces	-	(Petzel, W., Oberschleißheim)

III.2. OLIGONUCLEOTIDES

All oligonucleotides used for PCR, RT-PCR and/or qPCR were purchased from Sigma-Genosys (Steinheim, Germany) and Eurofins MWG Operon (Ebersberg, Germany). The sequences and the application of all oligonucleotides are listed in **Table 7**.

Table 7: Oligonucleotides used in this study

Primer	Sequence [5´→3´]	Application
GapA_for(756)	gttgtcgctgaagcaactgg	DNase treatment control; target is *gapA* (Blumer et al., 2005)
GapA_rev(757)	agcgttggaaacgatgtcct	DNase treatment control; target is *gapA* (Blumer et al., 2005)
16SRNA_for	aactgagacacggtccagact	Control in qRT-PCR; target is 16S rRNA
16SRNA_rev	ttaacgcttgcaccctccgt	Control in qRT-PCR; target is 16S rRNA
27f	gagtttgatcctggctca	Control in qRT-PCR; target is 16S rRNA
798r	ccagggtatctaatcctgtt	Control in qRT-PCR; target is 16S rRNA
Sf0379	tcagcctgcagattagcgacgt	Screening for *upaB*
Sr0379	tatgtccggactgcaatggtca	Screening for *upaB*
F3LC0433	caatgccaatggcgatttcga	Screening for *upaC*
R3LC0433	ggtcaagctgttcggcgcaacat	Screening for *upaC*
Sf2276	gctgtcagtttcaaggttgg	Screening for *upaI*
Sr2276	tcttgccggaacgggagtcat	Screening for *upaI*
F2LC3703	gggtagtcaatccaatgcaaacggt	Screening for *upaJ*
R1LC3703	ccagggtatcaacgtccgcgttca	Screening for *upaJ*
0379cF	tgctctagaaggaattgttatggagaatttct	Amplification of *upaB* from *E. coli* 536
0379cR	cccaagcttagtcgacaggggaaccgactgct	Amplification of *upaB* from *E. coli* 536
0433cF	tgctctagaaggaattgttatgcactcctgga	Amplification of *upaC* from *E. coli* 536
0433cR	ccgctcgaggcccgtcaaatccttgacgggca	Amplification of *upaC* from *E. coli* 536
2276cF	tgctctagaaggaattgttatgaatatgcgga	Amplification of *upaI* from *E. coli* 536
2276cR	cccaagcttcctgataaggcgtttacgccgca	Amplification of *upaI* from *E. coli* 536
3703cF	tgctctagaaggaattgttatgaacaaaatat	Amplification of *upaJ* from *E. coli* 536
3703cR	cccaagctttgctgaatcaccccgtaggcct	Amplification of *upaJ* from *E. coli* 536
Fp0379Fless	gcggtatcaactacaccggttacattgg	Mutagenesis of *upaB* with FLAG® tag
Fp0379Rflag	cttatcgtcgtcatccttgtaatcgttatcag	Mutagenesis of *upaB* with FLAG® tag
Fless0433F	acgaccgatttagtttggccgtatga	Mutagenesis of *upaC* with FLAG® tag
Flagprimer433R	cttatcgtcgtcatccttgtaatcggtgttgt	Mutagenesis of *upaC* with FLAG® tag
Fp2276Fless	cagggatatgatatcaaagcgagctgtcagg	Mutagenesis of *upaI* with FLAG® tag
Fp2276Rflag	cttatcgtcgtcatccttgtaatcacatgaat	Mutagenesis of *upaI* with FLAG® tag
Fp3703Fless	gcgcttgatggtggtggggctagcg	Mutagenesis of *upaJ* with FLAG® tag
Fp3703Rflag	cttatcgtcgtcatccttgtaatcggtcgatg	Mutagenesis of *upaJ* with FLAG® tag
RT_79_F	ctccaccatcacagctcaa	quantitative RT-PCR of target *upaB*
RT_79_R	accgccattaacaacaaca	quantitative RT-PCR of target *upaB*
RT_33_F	gttgggtgatgtcgagtt	quantitative RT-PCR of target *upaC*
RT_33_R	ggccggttgaatagaagaat	quantitative RT-PCR of target *upaC*
RT_76_F	ggcgatattgtggtggaag	quantitative RT-PCR of target *upaI*
RT_76_R	aggtggtgaaatcagagag	quantitative RT-PCR of target *upaI*
RT_03_F	agcacaacacaacgcaaaa	quantitative RT-PCR of target *upaJ*
RT_03_R	gcgcctctcccacattat	quantitative RT-PCR of target *upaJ*

III.3. CHEMICALS AND ENZYMES

All chemicals and enzymes used in this study were purchased from the following companies:

AppliChem (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany), Dianova (Hamburg, Germany), Difco (Augsburg, Germany), GE Healthcare/Amersham Biosciences (Freiburg, Germany), Gibco (Eggenstein, Germany), Invitrogen (Karlsruhe, Germany), MBI Fermentas (St.Leon-Roth, Germany), Merck (Darmstadt, Germany), New England Biolabs (Frankfurt am Main, Germany), Oxoid (Wesel, Germany), PAA (Cölbe, Germany), Roche Diagnostics (Mannheim, Germany), Roth (Karlsruhe, Germany), Serva and Sigma-Aldrich (Taufkirchen, Germany).

The following commercial kits were used:

- Plasmid Mini and Midi kit, QIAGEN (Hilden, Germany)
- QIAquick MinElute PCR purification kit, QIAGEN (Hilden, Germany)
- QIAquick MinElute Gel extraction kit, QIAGEN (Hilden, Germany)
- RNeasy kit, QIAGEN (Hilden, Germany)
- ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, Applied Biosystems (Foster City, USA)
- ECL™ Direct Acid Labeling and Detection System, and ECL™ advance system, GE Healthcare/Amersham Biosciences (Freiburg, Germany)
- OpArray Hybridization Buffer Kit, Operon (Cologne, Germany)
- Agencourt AMPure XP Kit, Beckman Coulter Genomics (Krefeld, Germany)

III.4. MEDIA, AGAR PLATES AND ANTIBIOTICS

All media were autoclaved for 20 min at 120 °C, if not stated otherwise. Supplements for media and plates were sterile filtered through a 0.22 µm pore filter and added after cooling down the media to <50 °C.

III.4.1. Media

DMEM High Glucose with L-Glutamine - Dulbecco's Modified Eagle Medium was purchased either from Gibco or Lonza.

LB medium (lysogeny broth) (Sambrook et al., 1989):

10 g Tryptone from casein

5 g Yeast extract

5 g NaCl ad 1 l dH_2O

M63 minimal medium:

Ingredient	stock	final concentration	for 800 ml
M63 salts	5x	1x	160 ml
$FeSO_4$	1 ‰	0.001 ‰	800 µl
$MgSO_4$	10 %	10 ‰	800 µl
Thiamin	0.2 %	0.5 ‰	2 ml
Glucose	20 %	0.4 %	16 ml
Casamino acids	10 %	1 %	80 ml
KOH	10 M pH 7		5.2 ml

5 x M63 salts

$(NH_4)_2SO_4$	15 mM	8 g
KH_2PO_4	100 mM	54.4 g add dH2O up to 800 ml and autoclave
$FeSO_4$	1 mg / 1 ml in H_2O and sterile filtration	
$MgSO_4$	10 g / 100 ml in H_2O and autoclaving	
Thiamin	20 mg / 10 ml in H_2O and sterile filtration	
Glucose	20 g / 100 ml in H_2O and autoclaving	
Casamino acids	10g / 100 ml in H_2O and autoclaving	
KOH	10 M in H_2O and autoclaving	

MM9 minimal medium (Kalinowski et al., 2000):

Ingredient	stock	final concentration	for 1000 ml
Tris (pH 6.8)	0.5 M	0.05 M	100 ml
Casamino acids	10 % (w/v)	1 % (w/v)	100 ml
Glucose	20 % (w/v)	0.4 % (w/v)	20 ml
$MgSO_4$	1 M	2 mM	2 ml
$CaCl_2$	1 M	0.1 mM	100 µl
Thiamine	10 mM	3 µM	300 µl
<u>10 x MM9 salts</u>			100 ml ad 1 l dH2O; sterile filtered

KH_2PO_4 3 g / l

NaCl 5 g / l

NH_4Cl 10 g / l

Modified for lactose growth curve: No casamino acids and glucose, but 0.2 % lactose (20 ml 20 % w/v lactose)

Milk whey

In collaboration with the Clinic for Ruminants of the Ludwig-Maximilians-University Munich (Oberschleißheim, Germany), we obtained raw milk from a generally udder healthy cow. After milking, raw milk was cooled to 4°C and processed to milk whey. For the preparation of milk whey, milk was centrifuged at 38,000 x *g* and 4°C for 30 min. The fat layer was removed with a spatula and the skim milk was decanted into a clean tube. A second centrifugation step followed as described above and the translucent supernatant was collected and stored at -80°C until use.

The described process removes fat, cell debris and partially depletes casein-peptones.

Milk whey from a cow challenged with LPS

This medium refers to milk whey (see above), but was obtained from a cow, which received an infusion of purified *E. coli* LPS into the udder. The LPS endotoxin binds to the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in many cell types, but especially in macrophages and B cells.

In immunology, the term "LPS challenge" refers to the process of exposing a subject to an LPS that may act as a toxin. For bovine neutrophils it has been reported that binding of LPS to membrane-bound CD14 causes release of TNFα and sepsis (Paape et al., 2003). Therefore LPS challenge is a proposed method to elicit immune response within in the udder.

III.4.2. Agar plates

LB agar plates: LB medium + 1.5 % (w/v) agar (Difco Augsburg, Germany)

III.4.3. Antibiotics

The isolates used in this study were not genetically manipulated. Hence, media and plates were not supplemented with any antibiotics. Instead, gentamycin (Sigma-Aldrich, Taufkirchen, Germany) was used in cell culture experiments to perform the gentamycin protection assay (section IV.5.3).

III.4.4. DNA Markers

In order to determine the size of DNA fragments in agarose gels, the "Generuler^{TM}" 1-kb DNA ladder, purchased from MBI Fermentas, was used (**Figure 5**).

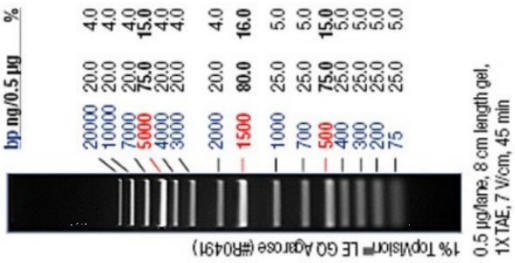

Figure 5: Generuler^{TM}, 1-kb DNA ladder

III.5. TECHNICAL EQUIPMENT

Accu-Jet Pro Pipette Controller	BrandTech (Wertheim, Germany)
AlphaImager ® EP	Alpha Innotech Corp. (Santa Clara, USA)
Autoclave	Tecnoclav biomedics Laborservice GmbH (Gießen, Germany)
Bio-Rad C1000 Thermal Cycler	Bio-Rad (Munich, Germany)
CFX96 RealTime System	Bio-Rad (Munich, Germany)
Biofuge 13R,	Heraeus Sepatech instruments (Langenselbold, Germany)
Bio-Rad Imager	Bio-Rad (Munich, Germany)
Concentrator Plus SpeedVac	Eppendorf (Hamburg, Germany)
Electrophoresis chambers	Bio-Rad (Munich, Germany)
Fluostar Omega ELISA Reader	BMG Labtech (Offenburg, Germany)
Gel documentation system	Intas (Göttingen, Germany)
Hettich Rotanta 460RS centrifuge	Hettich Lab Technology (Tuttlingen, Germany)
HiScanSQ NGS Sequencer	Illumina (San Diego, USA).
Camera, Ixus 500	Canon (Krefeld)
Refrigerated centrifuge, Megafuge10R	Heraeus Sepatech instruments (Langenselbold, Germany)
Refrigerated benchtop centrifuge,	
MultiNA microchip electropho. System	Shimadzu (Duisburg, Germany).
Lab901 TapeStation for NGS SampleQC	Agilent Technologies (Waldbronn, Germany)
Micropipettes	Eppendorf (Hamburg, Germany)
Microtiter plates, 96 Well	Sarstedt (Nürnbrecht, Germany)
Microtiter plates, 96 Well	Greiner (Solingen, Germany)
Mains unit, Mighty slim™, SX250	Hoefer (San Francisco, USA)
PCR-Thermocycler, T3	Biometra (Göttingen, Germany)
Precellys Homogeniser	Peqlab (Erlangen, Germany)
Pipettes	Eppendorf (Hamburg, Germany)
	Gilson AG (Mettmenstetten, Switzerland
Shaking incubators	B. Braun Biotech (Melsungen, Germany)
	GFL (Burgwedel, Germany)
	New Brunswick Scientific/ Eppendorf (Hamburg, Germany)
	Infors-HT (Bottmingen, Switzerland)
Sequencer	Applied Biosystems (Foster City, USA)
Sterile work bench	Nuaire (Plymouth, USA)
Sterile filters, 0.22 µm	Millipore (Schwalbach, Germany)
Spectrophotometer, ND-1000	Peqlab (Erlangen, Germany)
Spectrophotometer, ND-2000	Peqlab (Erlangen, Germany)
Thermo incubator	Liebisch (Bielefeld, Germany)
	Eppendorf (Hamburg, Germany)
Thermoshaker incubator	Peqlab (Erlangen, Germany)
Tabletop centrifuge	Heraeus sepatech instruments (Langenselbold, Germany)
	Eppendorf (Hamburg, Germany)
Ultraspec 2100 Pro and	
Ultraspec 3100 Pro	
Spectral Photometer	GE Healthcare (Munich, Germany)
UV-chamber	Bio-Rad (Munich, Germany)
Vortexer	GFL (Burgwedel, Germany)

Water bath	Memmert (Schwabach, Germany) GFL (Burgwedel, Germany)

III.6. Software

All software, databases and online resources used in this study are listed in **Table 8**.

Table 8: Software, databases and online resources

Resource	Application
Acuity 4.0 software (Molecular Devices, Sunnyvale, USA)	Statistical validation and further analysis
Artemis (Rutherford et al., 2000)	Genome annotation
Artemis Comparison Tool (Carver et al., 2005)	Genome comparison
BBF, β-barrel finder, (Zhai and Saier, 2002)	Detection of outer membrane proteins
BLAST (Altschul et al., 1990; Camacho et al., 2009)	Basic Local Alignment Search Tool
bROC Version 2 (BioFormatix, San Diego, USA) (BioFormatix, 2010)	Discovery of differentially expressed genes (DEG) in RNA-Seq experiments
CFX Manager™ Software Version 3.0 (Bio-Rad, Munich, Germany)	Experiment setup and data analysis software for CFX96™
CLC Genomics Workbench (Aarhus, Denmark)	Read mapping and transcriptomic analyses
Cluster 3.0 (Eisen et al., 1998)	Hierarchical clustering
FastQC Version 0.10.1 (Andrews, S.) http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	Quality control tool for high throughput sequence data
GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, USA)	Scanning of DNA microarrays
Inkscape Version 0.48.4.1 (Inkscape Community)	Vector graphic editing
jSpecies Version 1.2.1 (Richter and Rosselló-Móra, 2009; Goris et al., 2007)	Prokaryotic genome similarity analysis Average Nucleotide Identity (ANI)
MUMmer 3.0 (Kurtz et al., 2004)	Genome comparison
Office2007 Excel software (Microsoft, Redmond, USA)	Statistical validation and analysis
R Version 3.0.1 (R Foundation) *http://www.R-project.org*	Statistical computing and analysis
SortMeRNA Version 1.8 (Kopylova et al., 2012)	Filtering of ribosomal RNAs in metatranscriptomic data
TreeView (Page, 1996)	Visualization of hierarchical clustering

IV. METHODS

IV.1. Working with DNA

Working with DNA required no extraordinary handling. In contrast to RNA work which required handling with special care in order to prevent contamination of the RNA samples with exogenous RNases.

IV.1.1. Isolation of chromosomal DNA

1 ml bacteria from an overnight culture were harvested by 4 min centrifugation at maximum speed in a tabletop centrifuge. Followed washing in 1 ml TNE buffer, cells were centrifuged for 4 min and resuspended in 270 µl TNE-X buffer. For lysis of the bacteria 30 µl lysozyme (5 mg ml^{-1}) were added and samples were incubated for 20 min at 37°C. Subsequently 15 µl of proteinase K (20 mg ml^{-1}) were added to the samples which were 2 h incubated at 65°C until. The solution should have become clear. The genomic DNA was precipitated by addition of 0.05 vol 5 M NaCl (15 µl) and 500 µl ice-cold ethanol and subsequently collected by centrifugation for 15 min. After washing two times with 1 ml 70% (v/v) ethanol, the DNA pellets were air-dried and redissolved in 100 µl 100 dH_2O (Clermont et al., 2000).

TNE	TNE-X
10 mM Tris	TNE + 1 % Triton X-100
10 mM NaCl	
10 mM EDTA	

IV.1.2. Precipitation of DNA with alcohol

The DNA was precipitated with ethanol and isopropanol.

Precipitation with ethanol was performed with 0.1 volumes 3 M Na-acetate (pH 4.8), which were added to the sample followed by the addition of 2.5 volumes ice-cold 100 % (v/v) ethanol. The DNA pellet was washed with 70 % (v/v) ethanol, air-dried and resuspended in dH_2O.

Precipitation with isopropanol was performed by addition of 0.7 volumes isopropanol. The samples were incubated at -80°C before at least 20 min of centrifugation by 13,000 rpm at 4°C. The DNA pellet was washed with 70 % (v/v) ethanol, air-dried and resuspended in dH_2O.

IV.1.3. Determination of nucleic acid concentration and quality

Nucleic acid concentrations were determined by using the NanoDrop® instrument which measures absorption at 260 nm. Absorption of 1.0 corresponds to 50 µg ml^{-1} double stranded DNA. The sample purity of the preparations was determined by measuring the absorption ratio of the respective sample. DNA samples were considered sufficiently pure when the ratio A260/A280 was higher than 1.8, respectively.

IV.1.4. Polymerase chain reaction (PCR)

A PCR-screening for the AT genes *upaB*, *upaC*, *upaI* and *upaJ* was performed by PCR. The primers were designed based on the specific α-domain-encoding nucleotide sequence of the respective autotransporter genes. By testing of various primers with chromosomal DNA as template multiple PCR products occurred. This problem was solved by selection of primers homologous to gene sections with a low prevalence of repetitive sequences and by usage of bacterial lysates as template DNA. Screening was performed using the primers listed in **Table 7**.

The 25 µl screening-PCR mix for one template colony-PCR sample was:

14 µl H2O
5 µl Q-Solution 5×
2.5 µl Buffer
1 µl MgCl2 [25mM]
0.5 µl dNTPs (10µl per dNTP + 60µl H2O)
0.5 µl Primer 1
0.5 µl Primer 2
0.15 µl Qiagen™ - TAQ-Polymerase
1 µl Template colony-PCR

Program:

1:	94°C	180s
2:	94°C	30s
3:	Tm°C	60s
4:	72°C	60s /kb -> 2 25-35cycles
5:	72°C	600s
6:	12°C	hold

IV.1.5. Sequence analysis (Sanger)

The nucleotide sequences of bacterial DNA were determined using fluorescent dye terminators (ABI prism BigDye terminator kit, Applied Biosystems) at the Institute for Molecular Infection Biology (*IMIB*), University of Würzburg.

The sequencing-PCR mix for one sample was:

30 ng PCR product (or: 0.5 µg plasmid DNA)
1.5 µl 10 pM primer
2 µl 5 x buffer (kit component)
2 µl premix (kit component) ad 10 µl ABI-H2O

The thermal cycling profile for the PCR reaction was: 40 cycles of denaturation at 96 °C for 30 s, annealing at 60 °C for 15 s, and extension at 60°C for 4 min, followed by final extension at 60 °C for 2 min. The sequencing products were purified by ethanol precipitation and analyzed in an ABI prism sequencer (Perkin Elmer).

IV.1.6. Separation of DNA fragments by gel electrophoresis

DNA fragments were separated on a 0.8 % and 1 % (w/v) agarose gels (1 × TBE buffer with 1 mM urea) by horizontal electrophoresis. Afterwards DNA fragments in the gel were stained in an ethidium bromide solution (10 g/ml) and the gels were photographed on an UV-transilluminator.

IV.2. Working with RNA

The work with RNA required handling with special care in order to prevent contamination of the RNA samples with exogenous RNases. Therefore gloves were worn throughout conduction of RNA experiments. Furthermore, RNase-free pipette tips and reaction tubes were used. For all buffers and solutions, water was treated over night with 0.1 % (v/v) diethylpyrocarbonate (DEPC) at 37 °C and autoclaved twice to remove remaining DEPC. For dissolving RNA in water, nuclease free water from the kits listed in section III.4. was used.

IV.2.1. Standard isolation of total RNA with Qiagen RNeasy Kit

RNA preparation was performed by using the RNeasy Mini kit (Qiagen) following the manufacturer's protocol. All subsequent steps of the RNeasy protocol were performed at room temperature. For RNA isolation bacteria were grown at 37 °C in either LB or DMEM medium until the optical density (OD_{600}) reached 0.15. For RNA isolation from LB cultures, bacteria were grown without agitation at 37°C in 25 ml until the OD_{600} reached 0.6.

Four ml of the bacteria culture in either LB or DMEM respectively were taken and centrifuged at 6000 rpm for 5 min. The supernatant was removed and pellets were resuspended in 4 ml of PBS and RNAprotect Bacteria Reagent™ (Qiagen) 1:1 (v/v), respectively. Samples were incubated at room temperature for 5 min and centrifuged at 6,000 rpm for 10 min. The collected bacterial pellets were either stored at -80°C or bacterial RNA was immediately isolated. Following immediate RNA isolation bacterial pellets were resuspended thoroughly in 100 µl of lysozyme-containing TE buffer (50 mg/ml) and incubated at 37°C for 5 s while vortexing every 2 min. The following steps of the protocol are consistent with those of the protocol supplied with the Qiagen RNeasy® Mini Kit.

IV.2.2. Isolation of bacterial total RNA from milk whey

Specific treatment allowed isolation of bacterial total RNA from milk whey spiked with *E. coli*. For this purpose 15 ml of prewarmed milk whey, inoculated with $1x10^9$ colony forming units (CFU)/ml, were statically incubated for 1 h at 37°C. After cultivation the degradation of transcripts was inhibited by addition of two sample volumes of the RNA stabilizing reagent RNAprotect Bacteria Reagent™(Qiagen), followed by 5 min incubation at room temperature (15-25°C). Bacteria were harvested by centrifugation and the casein-peptone protein content was reduced by three times washing with 50 v/v % RNAprotect Bacteria Reagent (PBS). In detail, the reagent-milk whey mixture separates into a translucent fluid and precipitated proteins. The fluid supernatant containing the

major portion of bacteria was transferred into a new tube and centrifuged at 5,000 x g and 4 °C for 10 min. The supernatant was then discarded and the pellet was washed with 30 ml 1:1 (v/v) 1 x PBS and RNAprotect Bacteria Reagent™ (Qiagen) by centrifugation with the previous settings. The obtained bacterial pellets were stored at -80 °C until further RNA isolation using the Qiagen RNeasy® Mini Kit. The bacterial pellets were resuspended thoroughly in 100 µl of lysozyme-containing TE buffer (50 mg/ml) and incubated at 37 °C for 5 s while vortexing every 2 min. Subsequently the manufacturer´s protocol was followed.

This protocol allowed bacterial RNA isolation from milk whey with sufficient amounts of high integrity RNA.

IV.2.3. Isolation of total RNA from bacteria upon cocultivation in cell culture

This protocol has been used for the assessment of the bacterial transcriptome upon bacterium-host interaction by cocultivation with MAC-T immortalized bovine mammary gland epithelial cells. For this purpose the principle of column based RNA isolation utilized by the Qiagen RNeasy® Mini Kit has to be considered. Nucleic acids are competitively binding to silica membranes in which the kind of nucleic acids binding to silica membranes is mainly influenced by the ratio of sample to salts to ethanol. Clogging of the columns and contaminations due to genomic DNA mainly derived from the eukaryotes can be prevented by the use of gDNA Eliminator spin columns (Qiagen). This type of column, in combination with the optimized chaotropic high-salt buffer, allows efficient removal of genomic DNA.

For total RNA isolation confluent 175-cm^2 cell culture flasks with 15 ml DMEM containing approximately 1.5 x 10^7 cells each were inoculated, with a multiplicity of infection (MOI) of 100, from bacterial overnight cultures (grown in DMEM). The cocultivation period was either 1 h or 3 h at 37 °C. After cocultivation the degradation of transcripts was inhibited by addition of two sample volumes of the RNA stabilizing reagent RNAprotect Bacteria Reagent™(Qiagen), followed by 5 min incubation at room temperature (15-25 °C). Both, the supernatant cell culture medium DMEM containing planktonic bacteria and the MAC-T cells with adhering and invasive bacteria were harvested by scratching the cell culture flask with a cell culture spatula. The samples of 15 ml culture (containing bacteria and eukaryotic cells) were taken and centrifuged at 6,000 rpm for 5 min. The supernatant was removed and pellets were resuspended in 4 ml of PBS and RNAprotect Bacteria Reagent™ (Qiagen) 1:1 (v/v), respectively. Samples were incubated at room temperature for 5 min and centrifuged at 6000 rpm for 10 min. The collected pellets were either stored at -80 °C or bacterial RNA was immediately isolated. Following immediate RNA isolation the pellets were resuspended thoroughly in 400 µl of lysozyme-containing TE buffer (50 mg/ml) and incubated at 37 °C for 5 s while vortexing every 2 min. The lysate was divided into four subsamples and then passed through a gDNA

Eliminator spin column (Qiagen) respectively. The following steps of the protocol were consistent with those of the protocol supplied with the Qiagen RNeasy® Mini Kit.

IV.2.4. RNA processing and quality control

In order to use isolated RNA as template for further analyses such as qRT-PCR, DNA microarrays and RNA-Seq, the total RNA had to be processed to ensure that it meets the requirements in purity and integrity.

DNase treatment and RNA purification

Contaminating DNA was removed from total RNA preparations by DNase I digestion. 15 µg RNA in a final volume of 85 µl were mixed with 10 µl 10 x DNase I buffer and 10 µl RNasefree DNase I (New England Biolabs). Samples were incubated for 1 h at 37 °C, followed by RNA cleanup using the RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. Therefore, 350 µl RLT buffer supplemented with 10 µl ß-mercaptoethanol and 250 µl 100 % (v/v) ethanol were added to the DNase-treated RNA samples. Samples were loaded to the supplied purification columns and briefly centrifuged. In order to prevent any residual buffer, the columns were transferred to fresh collection tubes and washed twice with 500 µl RPE buffer. The purified RNA was then eluted from the column in 30 µl nuclease-free water. Elimination of DNA was verified on a CFX96 real-time PCR machine (BIO-RAD, Munich, Germany) using SSoFast™ EvaGreen® Supermix (BIO-RAD) with the primers listed in **Table 7**. Therefore 2 µl of the DNase-treated RNA were used as a template in a PCR reaction with primers binding either within the coding sequence of the *gapA* or *frr* gene. The DNase treatment was considered successful if no product could be amplified from the RNA samples. Residual DNA was removed by repeating this protocol.

RNA quality control

We evaluated the quality of RNA using the RNA integrity number (RIN) as generated by Agilent Technologies' 2100 Expert Software. The RIN allows for an objective and standardized assessment of RNA. It is automatically generated and the number takes into account several features commonly ascribed to overall RNA quality (Schroeder et al., 2006).

RNA concentrations were determined using the NanoDrop® instrument, which measures absorption at 260 nm. Absorption of 1.0 corresponds to 40 µg ml^{-1} RNA. The sample purity of the preparations was determined by measuring the absorption ratio of the respective sample. For this, RNA samples were considered sufficiently pure when the ratio A260/A280 was higher than 2.0.

The RNA integrity was determined by capillary electrophoresis using either the Agilent 2100 Bioanalyzer instrument or the Lab901 TapeStation for NGS Sample QC following the supplied protocols. RNA integrity values (RIN) > 7.0 were considered to meet the requirements for expression analyses (Schroeder et al., 2006; Imbeaud et al., 2005; Wilkes et al., 2010).

IV.2.5. cDNA synthesis by reverse transcription (RT)-PCR

For further analyses the RNA has to be reversely transcribed by a RNA-dependent DNA polymerase in order to synthesize cDNA in a subsequent amplification by PCR. For the purpose of cDNA synthesis, the Superscript III reverse transcription kit (Invitrogen) was used. 2 µg of total RNA in a final volume of 10 µl were mixed with 1 µg of random hexamer primers (Amersham Biosciences). Primer annealing was carried out at 65 °C for 5 min. After 5 min cooling, 9 µl of a reverse transcription mixture were added to the samples. The RT-PCR-mix for on sample was pipetted as below:

1 µl 25 mM deoxynucleotide mix

1 µl 0.1 M dithiothreitol (DTT; kit component)

4 µl 5 x first strand buffer (kit component)

1 µl 40 U µl-1 RNase OUT recombinant RNase inhibitor (Invitrogen)

1 µl 200 U µl-1 Superscript III reverse transcriptase (Kit component)

The cDNA synthesis was performed at 52 °C for 60 min, followed by heat inactivation of the transcriptase at 70 °C for 15 min.

IV.2.6. Quantitative Real-Time PCR

The quantitative Real-Time PCR (qRT-PCR) was used to assess gene expression of specific genes. This method utilizes PCR to amplify gene transcripts in presence of the SYBR Green I dye (Bio-Rad). qRT-PCR detects the amount of product quantitatively and in real time enabled by fluorescent dye that intercalates into double stranded DNA and emits signals collected by the optical camera within the CFX96 RealTime System (Bio-Rad). The transcript levels were computed by means of cycles needed to traverse the fluorescent signal threshold (CT) line by (CFX Manager™ Software, Bio-Rad). The primers for the selected genes were designed by the following parameters: product length range from 190 to 300 nt; annealing temperature 57 - 59 °C (by either Fast PCR or CLC Workbench - software) matching at least 90 % amplification efficiency. The cDNA samples derived from the RT-PCR were 100-fold diluted in dH_2O and the qRT-PCR-mix for one reaction was pipetted as below:

12.0 µl cDNA

12.5 µl SYBR Green Mix 2 ×

0.25 µl Primer 1 (10 µmol)

0.25 µl Primer 2 (10 µmol)

The thermal cycler was programmed as follows:

3 min 95 °C

30 s 95 °C

30 s 60 °C – 40 cycles

20 s 72 °C

30 s 95 °C – 57 °C (melting curve)

All PCR reactions were done in triplicates and primers either for the gene *frr* (ribosome recycling factor) or *rrnB* (encoding for 16 S RNA) were used as an internal control.

IV.3. Transcriptome analysis by DNA-microarrays

Expression profiling is a technique to study the relative amounts of all transcripts at a given time of sample collection. It thereby allows monitoring the expression level of every single gene detectable by the array with restriction to small RNAs which are lost during total RNA extraction and purification by the Qiagen RNeasy Kit. This protocol follows the main protocol established in the working group of Dobrindt and colleagues

Array Layout

In order to comprehensively and quantitatively assess the transcriptional response of bacteria upon cocultivation with eukaryotic cells by expression profiling, customized oligonucleotide glass microarrays (Operon Biotechnologies, Inc.) were employed. A single Operon *E. coli* Custom 55156017 array contains 10,816 longmer oligonucleotide probes covering the complete genomes of six *E. coli* strains (6 genomes and four plasmids).
The number of open reading frames (ORFs) or genes represented is as follows:

4,269 ORFs of non-pathogenic *E. coli* K-12strain MG1655,
5,306 ORFs of enterohemorrhagic *E. coli* O157:H7 strain EDL933,
5,251 ORFs of enterohemorrhagic *E. coli* O157:H7 strain Sakai,
5,366 ORFs of uropathogenic *E. coli* strain CFT073,
322 ORFs of uropathogenic *E. coli* strain 536,
448 ORFs of uropathogenic *E. coli* strain UTI89,
3 genes of EHEC plasmid OSAK1,
10 genes of EHEC plasmid pO157_Sakai,
97 genes of EHEC plasmid pO157_EDL933 and UPEC plasmid pUTI89.

In addition, the array comprises also a number of positive and negative controls. Each probe contains an amino linker at the 5'- end. Probes are spotted as single spots in 32 blocks (4 columns, 8 rows), each block with 18 columns x 19 rows.

Sample preparation

Total RNA samples were prepared from bacteria upon cocultivation with MAC-T immortalized bovine mammary gland epithelial cells followed by DNase treatment, as described in sections IV.2.3 and IV.2.4. All DNA microarray experiments were done in triplicates including the overnight culture.

For cDNA labeling all procedures involving fluorescent dyes had to be done quickly and by avoiding exposure to light because of photosensitivity. Reverse transcription was performed using SuperScript IIITM reverse transcriptase (Invitrogen) and the fluorescently labeled nucleotides Cy3- and Cy5-dCTP (GE Healthcare). All solutions were prepared with DEPC treated water. For primer annealing, the annealing mix for one reaction was prepared as below:

10 µg total RNA

1 µg hexamer oligos

ad H_2O (DEPC treated) to a total volume of 15 µl

The annealing mix was heated for 10 minutes at 70 °C, then cooled down to room temperature for 5 min followed by brief centrifugation to spin down condensate water. The reaction mix for one reaction was prepared as below:

8 µl 5 x first strand buffer

4 µl 0.1 x DTT

4 µl Nucleotide mastermix

1 µl RNaseOut

1 µl SuperScript III™ (200 U/µl)

4 µl RNase free H_2O

22 µl reaction mix and 15 µl annealing mix was immediately pipetted into a 50 µl PCR reaction tube. Either 4 µl Cy3- or Cy5-dCTP (1 mM) was added and the total mix of 41 µl was subsequently incubated for 1 h at 46 °C. After 25 min, another 1 µl of SuperScript IIITM reverse transcriptase (200 U/µl) was added. After incubation the reaction was stopped by addition of 5 µl EDTA (500 mM) and 10 µl NaOH (1 M) was added to hydrolyze the RNA following incubation at 65 °C for 15 min. The reaction mixture was cooled down to room temperature and 25 µl Tris-HCl (1M, pH 7.5) was added.

The labeled targets were purified using the Qiaquick PCR Purification Kit (Qiagen) following the manufacturer's instructions with minor changes. Briefly, 5 volumes of PB buffer were added and each sample was applied to a Qiaquick column and centrifuged at max speed for 30 s. The column was washed with 700 µl PE buffer and dried by centrifugation. cDNA was eluted in 30 µl dH_2O and collected in a 1.5 ml Eppendorf tube. Of this, 1 µl was taken to control for quality and dye incorporation and quantification using the NanoDrop photometer. The remaining labeled cDNA was dried using a Concentrator Plus SpeedVac (Eppendorf). The cDNA pellet was resuspended in 2 µl dH_20.

Array pre-hybridization

The Array slides were cleaned by compressed air from dust particles and pre-hybridized in pre-warmed OPArray Pre-Hyb solution at 42 °C for 1 h. During that time Wash Solution 1 was prepared by diluting OpArray Wash B 1:40 (v/v) with chromatography grade ROTISOLV® HPLC water (Carl Roth GmbH). ROTISOLV® HPLC water was also used for all further wash solutions and array rinsing with water (further announced as dH_2O). Arrays were washed for 5 min at 20-25 °C and immediately rinsed with dH_2O for 30 s. This step was repeated twice. The slides were dried in 50 ml Falcon tubes with a hole in the bottom by centrifugation at 1200 rpm for 10 min. Residual liquid was removed by compressed air.

Array hybridization

The hybridization chamber was rinsed with sterile dH_2O and dried thoroughly. In the four corners of the chamber, 15 µl of sterile dH_2O were added in order to keep the humidity during hybridization time. The OpArray was placed into the chamber with the DNA side up (barcode side up) and the spotted area was covered with a LifterSlip (Thermo Scientific). Cy5- and Cy3-labelled cDNA targets were mixed with 36 µl of OpArray Hyb Buffer, denatured at 65 °C for 5 min and then applied slowly to one end of the LifterSlip in order to disperse across the OpArray surface. The hybridization chamber was closed and the arrays were incubated in a water bath at 42 °C for 14-16 h.

Post-Hybridization washing

For Post-Hybridization washing the following solutions were prepared:

Wash Solution 2:

50 ml OpArray Wash A

25 ml OpArray Wash B

Bring Wash Solution 2 final volume to 500 ml with sterile dH_2O

Wash Solution 3:

50 ml OpArray Wash A

Bring Wash Solution 3 final volume to 500 ml with sterile dH_2O

Wash Solution 4:

5 ml OpArray Wash A

Bring Wash Solution 4 final volume to 500 ml with sterile dH_2O

After hybridization, the arrays were washed in pre-warmed Wash Solution 2 at 42 °C for 10 min, transferred to Wash Solution 3 and shaken for another 10 min at RT. Subsequently, arrays were washed twice in Wash Solution 4 at RT for 5 min. Alike in the pre hybridization step, the microarrays were dried by centrifugation in 50-ml Falcon tubes for 10 min. The arrays were scanned subsequently.

Array Scanning

After hybridization, the array slides were scanned using a GenePix Model 4000B Microarray Scanner (Axon Instruments Inc., Union City, USA) with a resolution of 5 µm pixel size. The excitation frequencies of the two lasers were 532 nm and 635 nm, respectively. The gain settings for the photomultiplier tubes were adjusted to use the entire dynamic range of the instrument and to get comparable fluorescence yields in both channels. Images of Cy3 and Cy5 signals were recorded as 2 layer 16bit TIFF files and analyzed using the GenePix Pro 6.0 software.

Data analysis

For each experiment, at least three independent hybridizations were performed. Signals of bad quality spots were removed when matching any of the following criteria:

Less than 70 % of foreground pixels were below background intensity plus 2 standard deviations in both channels. The signal to noise ratio were below 3 in both channels. The difference between ratio of medians and regression ratio exceeded 20 % in one of the channels. The remaining intensities were saved as *.gpr type output data files. For statistical validation and further analysis the Acuity 4.0 software (Molecular Devices, Sunnyvale, USA) was used. For all data, the local background was subtracted from the intensity values of each spot on the array and normalized by both linear ratio-based methods and non-linear lowess including print-tip groups. For statistical significance, the one sample t-test was applied and the resulting data set was exported to the Office2007 Excel software (Microsoft, Redmond, USA). Mean substance values (<-1 and >1) and a p value of 0.05 were employed as cut-off values. Hierarchical clustering of genes for visualization of expression patterns was performed with the CLUSTER software (Eisen et al., 1998). The data output was displayed with the software TREEVIEW (Eisen et al., 1998).

For data analysis, a cut-off value of 1.7 was set although the threshold value sometimes referred to in literature is twofold (Wildsmith and Elcock, 2001; DeRisi et al., 1997). However, it has been shown (Pérez-Amador et al., 2001), that a lower cut-off ranging from 1.4 to 1.74 can be used reliably if the results are reproducible in more replicates.

For the final evaluation of differential gene expression, the obtained expression data were aligned to *E. coli* 1303 and ECC-1470 genome data (A. Leimbach, unpublished data).

IV.4. Transcriptome analysis by RNA-Seq

In order to comprehensively and quantitatively assess the transcriptional response of bacteria surviving and growing in milk whey we utilized RNA-Seq. RNA-Seq is a technology that makes use of next-generation sequencing (NGS) to reveal a whole transcriptome by sequencing of cDNA prepared from total cellular RNA. The gene expression can be followed by RNA-Seq to the extent at which the corresponding mRNA sequence is retrieved.

Sample preparation for RNA-Seq

E. coli strains were cultured overnight in LB media at 37 °C with agitation. Bacteria were harvested by centrifugation at 5,000 rpm and subsequently washed with PBS. Afterwards 15 ml of prewarmed milk whey were inoculated with 1 x 10^9 CFU/ml of bacteria and statically incubated for 1 h at 37 °C.
Growth was monitored by determining viable CFU by serial 10-fold dilution of cultures in PBS and plating onto LB agar. Plates were incubated overnight at 37 °C before colonies were counted.
Bacterial total RNA was isolated from *E. coli* strains, likewise described in section IV.2.2, upon cultivation either in milk whey or various media as control. Contaminating DNA was removed from total RNA preparations by DNase I digestion to below levels detectable by PCR. Finally, the RNA was purified and controlled for quality and integrity as described in section IV.2.4.

cDNA synthesis

The cDNA synthesis was carried out by vertis Biotechnologie AG (Freising, Germany). Briefly, the RNA samples were treated with terminator-5'-phosphate-dependent exonuclease (Epicentre Biotechnologies) to deplete processed RNAs. TEX degrades RNAs with a 5' monophosphate (i.e., processed transcripts) but not with a 5' triphosphate or 5' CAP structure (i.e., primary, unprocessed transcripts). Afterwards the RNA samples were fragmented by ultrasound treatment (4-6 pulses of 30 s at 4 °C). Samples were poly (A)-tailed using poly (A) polymerase and were then treated with tobacco acid pyrophosphatase (TAP) (Epicentre Biotechnologies) for 1 h at 37 °C to generate 5'-monophosphates for linker ligation. After TAP treatment, a RNA oligonucleotide was ligated to the 5'-monophosphates of the RNA molecules. First-strand cDNA synthesis was performed using an oligo (dT)-adapter primer and M-MLV H- reverse transcriptase. The resulting cDNAs were PCR-amplified to about 20-30 ng/µl using a high fidelity DNA polymerase (with cycle numbers according to sample concentration). The primers used for PCR amplification were designed for TruSeq sequencing according to the instructions of Illumina (San Diego, USA). The supplied barcode sequences were attached to the 5'-ends of the cDNAs. The cDNAs were purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics) and the cDNA pools were analyzed by capillary electrophoresis on a MultiNA microchip electrophoresis system (Shimadzu, Duisburg, Germany).

Sequencing

The sequencing of the cDNA pools was carried out at the core facility of LIFA (Leibniz-Institut für Arteriosklerosеforschung, Münster, Germany). The prepared cDNA pools were sequenced in a flowcell on the HiScanSQ (Illumina) with 101 cycles plus 7 cycles index read using the Illumina protocol 1.5. The sequences of the obtained sequencing reads were demultiplexed and barcode trimmed.

IV.4.1. Handling of RNA-Seq data

rRNA depletion and quality control

The raw reads of 101 bp of 23S and 16S rRNA were depleted using SortMeRNA 1.8 software with settings: r = 0.25, L = 18. The sequence reads were quality controlled with FastQC to assess the Sanger variant Phred scores (**Figure 24**). Phred scores *Q* assess the reliability of a base call and are defined as a property that is logarithmically related to the base calling error probabilities *P*:

$Q_{Sanger} = -10 \log_{10} P$

Afterwards, the sequencing data were submitted to the CLC Genomics Workbench v6.0.3 and quality trimmed with a cut-off Phred score of 20. Additionally, the sequence reads were truncated from the 90th base of the sequences and the resulting sequences were filtered by length and sequences short than 15 nt were discarded. Detailed quality statistics files were generated and summarized in Sequencing QC Reports.

Read mapping

The remaining reads were aligned to the reference sequences using the CLC bio read mapping algorithm. The reference sequences for both isolates *E. coli* 1303 and ECC-1470 were acquired by 454 Roche sequencing technology (A. Leimbach, unpublished) and the sequence reads of our samples have been generated with the Illumina platform. The 'embl' genome data files and 'gene only' annotations were used. The number of CDS (coding sequences) and genes (/pseudo genes) determined for the *E. coli* 1303 and the *E. coli* ECC-1470 genome are as follows:

Acute mastitis isolate Ec 1303	chromosome:	4725 CDS	4903 (74) genes
	F-plasmid:	120 CDS	122 (2) genes
	cryptic plasmid:	6 CDS	6 (?) genes
	P1-phage:	120 CDS	124 (1) genes
Persistent mastitis isolate ECC-1470	chromosome:	4430 CDS	4582 (42) genes
	F-plasmid:	120 CDS	121 (1) genes

The CLC bio read mapping parameters were set as follows:

Reads were neither mapped to flanking regions of coding sequences (CDS) such as intergenic regions, nor extended annotated gene regions such as flanking upstream or downstream residues.

The minimum length fraction was set to 0.9 which means that at least 90 % of the nucleotides had to be aligned to the reference.

The minimum similarity fraction specifies how exact the matching part of the read should be. This parameter was set to 0.8, and with the setting for the length fraction at 0.9, it means that 90 % of the read should have aligned with 80 % similarity in order to include the read.

Strand-specific alignment was selected at forward orientation. This option specifies whether the reads were attempted to map only in their forward orientation what is appropriate when a strand-specific protocol for read generation has been used. It also allowed assignment of the reads to the right gene in cases where overlapping genes were located on different strands.

Reads that matched to more distinct places in the reference sequence than the 'Maximum number of hits for a read' set at 10 were not mapped. If a read matches to multiple distinct places, but below the specified maximum number, it will be randomly assigned amongst the matching regions. The random distribution is weighted proportionally to the number of unique matches to the genes normalized by length. The gene that has the highest number of unique matches will thus get a greater proportion of the 10 reads.

To ensure that genes with no unique matches have a chance of having multi-matches assigned to them, 1 will be used instead of 0 (for their count of unique matches).

Reads mapped to rRNA genes (*rrs*, *rrl* and *rrf*) (Neidhard and Curtiss, 1996) and reads not mapped under the mentioned parameters were removed from further analysis. Mapping statistics were summarized in a Mapping Report (**Table 12** , page 116).

Mapped reads were visualized and subsequently analyzed using the CLC Genomics Workbench 6.0.3 and subsequent analyses were performed using either Microsoft Excel 2007 or R version 3.0.1.

Quantification of gene expression – transformation and normalization

Expression values for each gene were estimated by the number of reads that mapped within each annotated coding sequence (CDS). In consideration of gene expression being analyzed between different RNA-Seq samples the number of gene reads (either total or unique, as stated in the results section page 115 to **Fehler! Textmarke nicht definiert.**) was selected and quantile normalized. In quantile normalization, the distribution of read counts per sample (sequencing depth) is matched to a reference distribution defined in terms of median counts across the samples to compare (Gupta et al., 2012; Bullard et al., 2010).

While normalized, expression values were $\log_2$-transformed to ensure that samples are comparable and to remove noise effects:

Fold Change = $\log_2 (I_A / I_B)$

I_A and I_B are meant to be the mean expression values measured for group A and group B, respectively. RNA-Seq data might contain null values and if not stated otherwise the data were unity shifted and $\log_2$-transformed:

Fold Change = $\log_2 [(I_A +1) / (I_B +1)]$

The variability and similarity across samples as well as the distribution of expression measures between different genes within a sample were analyzed by expression values which take the gene length into account. Therefore the standard reads per kilobase of gene model per million mapped reads (RPKM) (Mortazavi et al., 2008) has been proposed as a useful metric that normalizes for variation in transcript length and sequence depth. Obtained expression measures were expressed as $\log_2$-transformed RPKM as they are normally distributed.

Whenever possible, the total read count of the respective transcript was used as expression measure since in most experiments a particular transcript was compared to its corresponding transcript in the reference sample. Obviously, the samples were quantile normalized before to define the default level. The explicit considerations on when to use what particular metric are discussed in the Results section.

Analysis of differential expression of genes (DEG)

Differential expression of genes (DEG) was identified using bootstrapped Receiver Operating Characteristic algorithm (bROC). bROC handles the instance of missing replicates by resampling (bootstrapping) the expression data to produce a larger number of simulated measurements that preserve the statistical properties of the original data. The ROC curve is supposed to be metric that ranks truly DEG ahead of non-DEG. This is evaluated by the area under ROC curve (AUC), as well as in terms of false discovery curves, depicting the number of false detections encountered while going through the list of genes ranked according to the evidence for differential expression (Soneson and Delorenzi, 2013).

The total read count of each gene was determined and both *E. coli* 1303 and ECC-1470 were treated as biological replicates per state/condition. Sample data from both the isolates at the respective condition were used for bROC analysis. For DEG discovery two group experiments were performed using the bROC algorithm on quantile normalized read counts. Following this, the data were $\log_2$–transformed and unity shifted (+1) because the data included null values as well. The transformed read counts for both isolates in either milk whey or LPS challenged milk whey were compared to the transformed read counts measured for incubation in DMEM (or milk whey) in order to determine the $\log_2$-fold change of expression. Genes with a discrimination score of CONF > 0.95 were considered to be differentially expressed.

IV.5. MAC-T Cell Culture Experiments

Conditions

The MAC-T immortalized bovine mammary gland epithelial cells were grown as described by Huynh et al., 1991 with modifications. The MAC-T cells were passed approximately 50 times prior to receipt for all assays. Briefly, MAC-T cells were grown on 175-cm^2 plastic tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM High Glucose with 4 mM L-glutamine) (Lonza) containing 10 % fetal bovine serum (FCS, Cölbe, Germany). During culture, cocultivation and adhesion assays, MAC-T cells were incubated at 37 °C in 5 % CO_2: 95 % air (v/v). Prior to passage, cells were washed with PBS (Gibco). Cells were released from plastic with trypsin solution (5 % trypsin in PBS) for approximately 10 min at 37 °C until cells were released. Trypsinization was stopped by addition of growth medium plus FCS. Cells were not exactly of the same passage for each assay (passage 48 ±2).

IV.5.2. Adhesion assay

The adhesive abilities of *E. coli* model mastitis isolates 1303 and ECC-1470 were comprehensively assessed in an adhesion assay (Dogan et al., 2006, modified). The MAC-T cells were seeded in 24-well tissue culture plates (Cellstars, Frickenhausen, Germany) and incubated overnight at 37 °C in 5 % CO_2: 95 % air (v/v). Approximately 2 x 10^5 MAC-T cells were present per well during the adhesion assay. The wells were infected with a multiplicity of infection (MOI) of 10. Therefore 2 x 10^6 bacteria of an overnight culture were co-cultivated with the MAC-T cells for 2 h. *E. coli* strain HB101 was used as a negative control. After three washing steps with PBS (pH 7.4), eukaryotic cells were lysed in 1 % Triton X-100 for 20 min. Attached bacteria were plated on LB-agar and enumerated following overnight incubation at 37 °C. The colonies were counted following overnight incubation. Adhesion was determined by the total number of colony-forming units (CFU) recovered per well. Each assay was run in triplicate and repeated once.

IV.5.3. Invasion assay

In addition to the adhesive abilities of *E. coli* model mastitis isolates 1303 and ECC-1470 their invasive abilities were assessed by the gentamicin protection assay (Dogan et al., 2006, modified).

Briefly, confluent monolayers of MAC-T cells (2 x 10^5 cells per well) were seeded in 24-well tissue culture plates (Cellstars) and incubated overnight at 37 °C in 5 % CO_2: 95 % air (v/v). Cells were infected with *E. coli* at an MOI of 10 as described in the adhesion assay except that after the initial 2 h infection period, cells were washed three times in PBS and then incubated for another 2 h with medium containing 100 mg/ml gentamicin (Sigma Aldrich) to kill any extracellular bacteria. The number of invasive bacteria in each well was determined as described above and the total number of

CFU recovered per well was expressed as percentage adhesion or invasion of the initial inoculum. Each assay was run in triplicate and repeated three times.

IV.5.4. Statistical analysis

All assay data were summarized using the mean of three or more replicates per experiment. Raw values from each experiment were used as input for the statistical analysis. To determine significant differences, between adhesion and inhibited adhesion or invasion of a single strain, the one tailed Student´s t-test was performed. Statistical significance was defined at $P < 0.001$.

If necessary, the significance between different strains was determined. Considering differences in the initial inoculum of the respective strains, an analysis of covariance (One-Way ANCOVA for Independent Samples) was performed. The ANCOVA used k = 2 independent samples, where the individual samples, (e.g. *E. coli* strain 1303 and ECC-1470), represent k quantitative or categorical levels of the independent variable; DV = the dependent variable of interest; and CV = the concomitant variable whose effects one wishes to bring under statistical control (e.g. distinct inoculum). Statistical significance was defined at $P < 0.001$.

V. RESULTS AND DISCUSSION

V.1. *E. coli* 1303 and ECC-1470 lack particular genes of virulence associated to mastitis isolates

Former studies referred to particular virulence genes (shown in **Table 9**.) as the most prevalent virulence genes of *E. coli* isolates from either clinical or subclinical bovine mastitis. Thereby the frequency and combinations of virulence genes varied greatly among both the different studies and the individual isolates investigated. The presence of these particular genes among the genomes of *E. coli* 1303 and ECC-1470 was evaluated (via BLASTn and BLASTp) and indicated in **Table 9**. In the genomes of both the mastitis-causing *E. coli* strains, particular genes associated with iron acquisition and particular fimbriae are not present. Additionally, the genes encoding the heat-stable enterotoxins and Shiga toxin as well as the genes encoding for the regulator of the locus of enterocyte effacement (LEE) and the K1 antigen are missing in both strains. There are, however, many publications on the absence of known virulence-associated genes of ExPEC and IPEC among *E. coli* mastitis isolates.

Table 9: The virulence factors associated with mastitis present in *E. coli* 1303 and ECC-1470

Symbol	Presence	Trait	Reference
east1	†	heat-stable enterotoxin I	Blum & Leitner, 2013
bor/iss	†	increased serum resistance	Suojala et al., 2011, Blum and Leitner, 2013
cs31a		CS31A fimbria	Fernandes et al., 2011
csgA	†‡	large subunit of curli fimbriae	Silva et al., 2013
eaeH	†‡	effector protein (intimin)	Kerro Dego et al., 2012
f17a		F17A fimbria	Ghanbarpour & Oswald, 2010
fimA	†‡	major subunit of the fimbriae type 1	Silva et al., 2013
fimH	†‡	minor component of type 1 fimbria	Fernandes et al., 2011
fliC	†‡	flagellin	Silva et al., 2013, Kerro Dego et al., 2012
irp2		iron regulatory protein 2 (yersiniabactin)	Suojala et al., 2011
iucD		siderophore biosynthesis protein (aerobactin)	Suojala et al., 2011, Fernandes et al., 2011
iutA		iron acquisition	Kerro Dego et al., 2012
kpsF		polysialic acid transport protein of K1 antigen	Fernandes et al., 2011
lee		LEE-encoded regulator	Kerro Dego et al., 2012
lpfA	‡	long polar fimbriae	Blum and Leitner, 2013 Dogan et al., 2012
ompC	†‡	outer membrane porin protein C	Kerro Dego et al., 2012
ompA	†‡	predominant cell surface antigen	Kerro Dego et al., 2012
papC		outer membrane usher protein of P fimbria	Suojala et al., 2011 Fernandes et al., 2011
SDH	†‡	respiratory chain activity	Kerro Dego et al., 2012
stb		heat-stable enterotoxin II	Fernandes et al., 2011
stx1		Shiga toxin 1	Bean et al., 2004
tolC	†‡	multidrug expulsion(e.g. Hly, bile, fusaric acid)	Kerro Dego et al., 2012

† present in *E. coli* 1303

‡ present in ECC-1470

Moreover, this indicates that there may be different ways to cause mastitis (Shpigel et al., 2008). A remarkable difference, that possibly impacts the outcome of phenotypic assays of the present study, is the presence of *east1* and *iss* in *E. coli* 1303 but not in ECC-1470. Additionally, the ECC-1470 genome but not that of *E. coli* 1303 contains the *lpfA* gene which was associated with adhesion of *E. coli* mastitis isolates (Dogan et al., 2012). A former study reported that *lpfA*, *east1* and *iss* were the three most prevalent virulence factors found in the set of *E. coli* mastitis isolates analyzed in this study (Blum and Leitner, 2013). In summary, *E. coli* 1303 and ECC-1470 differ in the prevalence and combination of virulence genes described in *E. coli* mastitis isolates.

Genome comparison of *E. coli* 1303 and ECC-1470

Do the differences in virulence gene content of *E. coli* strains 1303 and ECC-1470 individually affect the fitness and survival of these isolates? To assess their overall genome sequence similarity, despite the differences in virulence gene content, the average nucleotide identity (ANI) has been determined using the jSpecies software (Richter and Rosselló-Móra, 2009; Goris et al., 2007). Both strains exhibited more than 98 % ANI although they were obtained from mastitis cases of different outcome. The ANI data are particularly interesting for interstrain comparison, since *E. coli* strain 1303 and ECC-1470 might originate from different environmental habitats (e.g. faeces, soil, herd transmission). One should not be deceived by the fact that >98 % ANI suggest a high genome sequence identity, species related. Actually, both strains have a similar genome size of approximately 5.0 Mb (*E. coli* 1303: 4,971 CDS) and 4.9 Mb ECC-1470: 4,550 CDS). Moreover, HGT contributed to genome plasticity of both strains as indicated by the presence of integrated prophages and an F-plasmid. Disregarding mobile elements such as prophages and genomic islands, the P1 bacteriophage plasmid and a cryptic plasmid were identified in *E. coli* strain 1303, but not in isolate ECC-1470 (Leimbach et al., unpublished data). Since the genome plasticity of *E. coli* affects the genome content and confers adaptation to different growth conditions and habitats, the 98 % ANI is reflecting similarity and marked differences at the same time. In comparison, the ANI of two different *Burkholderia cenocepacia* strains isolated from different habitats (i.e. from a cystic fibrosis patient or from soil) was reported to be 99.8 % (Yoder-Himes et al., 2009). Thus, given the fact that *E. coli* 1303 and ECC-1470 are at least able to cause mastitis, the ANI of these two *E. coli* mastitis strains is quite low. To address the question whether de-regulated and phenotypically accessible determinants might play a role during host cell contact, adaptation and survival in the presence of host cells or in milk whey, the transcriptome of these strains as well as their fitness and competitiveness has been studied under suitable conditions.

V.2. From raw milk to milk whey

We aimed at the comprehensive analysis of the transcriptional response of the bovine mastitis isolates *E. coli* 1303 and ECC-1470 under environmental conditions mimicking initial stages of pathogenesis. Therefore, we have tried to cultivate the investigated strains in raw milk and then isolate RNA from these bacteria grown in raw milk. Additionally, we performed growth and fitness assays in milk to identify phenotypic fitness- and virulence properties of mastitis isolates.

Unfortunately, transcriptome analysis requires isolation of bacterial mRNA from raw milk of mastitic cows within minutes. Furthermore, mRNA has a short half-life due to enzymatic degradation which results in loss of transcripts. It has been shown that degraded mRNA does not rule out the performance of comparative gene expression analysis, as long as the samples being compared are of equal quality (Auer et al., 2003; Imbeaud et al., 2005). Another problem is that alterions of gene expression levels in response to the handling and processing of the sample, cannot be prevented.

To minimize such unwanted effects, bacterial RNA can be stabilized and protected against degradation by the addition of RNA Protect Bacterial agent (Qiagen) before the bacterial cells are lyzed. Assuming that this is not easily applicable in milk, we comprehensively tested different experimental setups to establish reliable bacterial gene expression analysis in milk. As a result of the studies described in this section, we finally experienced that milk is an inappropriate growth medium to analyze either the bacterial transcriptome or phenotypic properties. Instead, we selected raw milk whey as a suitable medium mimicking *in vivo* growth conditions.

V.2.1. Why milk is hard to deal with

Understanding the biochemical basics of milk is essential if experiments are planned to be conducted in this medium. Milk is an emulsion or colloid of butterfat globules within a water-based fluid. Each fat globule is surrounded by a membrane consisting of phospholipids and proteins; these emulsifiers keep the individual globules from joining together into noticeable grains of butterfat and also protect the globules from the fat-digesting activity of enzymes found in the fluid portion of the milk. In non-homogenized cow milk, the fat globules have an average diameter of about four micrometers (Jost, 2000).

When we conducted first cultivations of *E. coli* isolates in raw milk, we found that CFU enumerations varied greatly. In contrast, fractions of homogenized milk showed consistent CFU counts (data not shown). We therefore assumed an accumulation of bacterial cells in fat globules as reported for Gram-positive bacteria. It was observed that in raw milk *S. aureus* formed clusters associated with fat globules, while in heat-treated milk (which results in homogenization of milk), bacterial agglutination did not occur (O'Flaherty et al., 2005). In this context, immunoglobulins have also been reported much earlier to bond bacteria to fat globules (Walstra et al., 1984). In summary, bacterial growth in raw milk was not traceable by spectrophotometry or CFU enumeration on agar plates and, therefore,

interfered with most phenotypic assays in raw milk. However, we were evaluating the possibility of transcriptome analysis in this medium.

The largest structures in the fluid portion of the milk are casein protein micelles: aggregates of several thousand protein molecules, bonded with the help of nanometer-scale particles of calcium phosphate. Each micelle is roughly spherical with a diameter of about 0.1 µm. There are four different types of casein proteins, and collectively they make up around 80% of the protein in milk, by weight. Most of the casein proteins are bound into the micelles (Jost, 2000). Another major protein fraction is provided by immunoglobulins. And at least, in case of mastitis, the increased amount of up to several millions of somatic cells per milliliter milk contributes to the difficulties arising in RNA isolation.

Altogether, the size, weight and biochemical composition of the raw milk components made it impossible to use purchasable RNA isolation kits based on columns as they will be blocked by the milk components. It also turned out, that the use of commercial RNA stabilizing products was impossible as they cause unmanageable reactions (e.g. denaturation of the casein) and subsequently hindered extraction of RNA of sufficient quality in proper time. Consequently, a specific solution to rapidly isolate the bacterial mRNA was required.

In order to isolate bacterial total RNA in appropriate amounts and quality for microbial transcriptome analysis, one major problem had to be solved: How to rapidly harvest the bacteria from raw milk without a considerable loss of bacteria? Taking the challenge, we were confronted with various techniques and auspicious options to overcome the difficulties of bacterial RNA isolation from raw milk.

Filtration and centrifugation

Several attempts of filtration and centrifugation were unsuccessful, because of the size and weight of casein protein micelles and somatic cells. The filters were blocked by the somatic cells and the protein. Fractionated centrifugation was time consuming and could not sufficiently separate bacteria from milk protein and somatic cells. In all approaches the fat globules accumulated on top of the liquid solution as a viscous phase. This fat layer was easily removable, but enough fat remained to interfere with the use of column-based RNA isolation kits.

Another possible approach was multi-stage filtration. It was assumed to remove somatic cells and protein micelles by consecutive filtration stages. Therefore, a vacuum pump together with the sequential use of Millipore™ filters made of nylon and hydrophobic PTFE with decreasing pore size was used to avoid clotted filter membranes. It turned out that the protein content exceeded the filters' capacity and in consequence increased processing time.

Non-column based RNA isolation

We tested non-column based RNA isolation protocols in order to standardize RNA isolation for mastitis *E. coli* transcriptome investigation. But even RNA extraction methods based on the "one-step phenol-chloroform extraction method for RNA-isolation" (Chomczynski and Sacchi, 2006) failed as well as approaches based on salt precipitation like the Biozyme MasterPure™ Purification Kit. Accordingly, using commercially available non-column-based RNA purification methods did not allow to purify RNA in sufficient amount with high integrity. Alternatively, we evaluated the use of magnetic bead-attached *E. coli*-specific antibodies to enrich bacteria from raw milk. The binding and pull down of the bead-bound bacteria, however, took too much time, and bacteria were not selectively bound.

RNA transcript amplification

The afore mentioned conventional methods of RNA isolation from raw milk provided mostly bad quality RNA but rarely bacterial RNA of sufficient quality in low amounts. It would have been possible amplify RNA from low amounts of high integrity RNA using T7-RNA-polymerase based *in vitro* transcription. Notably, this method requires the usage of random primers and thereby bias may compromise the quantification of transcripts in transcriptome analysis.

So far the methodological potential to acquire bacterial RNA has been exploited.

Clarifying solution

We also tried to optimize bacterial RNA isolation from raw milk by "clarifying solution". This approach aimed at a reduced duration time of the bacterial isolation and rapid RNA stabilization when added to raw milk. To avoid aggregated casein protein micelles, a chelator was used. Because of its role as a chelating agent EDTA has been chosen to sequester metal ions such as Ca^{2+}. Thus, EDTA binds Ca^{2+} ions and prevents that calcium phosphate promotes formation of casein protein micelles. Casein protein will then remain in the supernatant during centrifugation.

Furthermore, to avoid that somatic cells will be pelleted together with bacteria by centrifugation, a detergent was added. Triton X-100 is a non-ionic surfactant, which can be used to permeabilize eukaryotic cell membranes. As a result, eukaryotic cells will disintegrate and their components remain in the supernatant upon centrifugation. Both, EDTA (0.25 M) and Triton X-100 (0.5 % v/v), have been combined in the clarifying solution.

In order to test the clarifying solution, 0.5 volumes were added to raw milk spiked with bacteria (1 x 10^7 CFU ml^{-1}) as bacterial counts of mastitis derived milk were reported to range from > 10^4 CFU ml^{-1} to 10^7 CFU ml^{-1} (Lues et al., 2010). Bacterial counts were determined by plating on LB-agar plates. Although this approach allowed subsequently to isolate bacterial RNA from raw milk, it also resulted in a considerable loss of bacteria. This was due to a severe reduction of bacterial survival when raw

milk was mixed with the clarifying solution (**Figure 6**). Control experiments where either raw milk or clarifying solution or both was replaced by PBS suggested, that raw milk provides a certain antimicrobial effect, but raw milk and clarifying solution that were mixed together completely decreased bacteria survival (**Figure 6**).

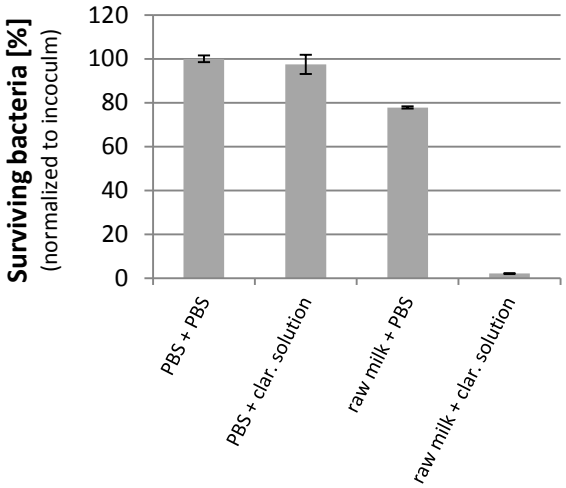

Figure 6: Impact of raw milk or clarifying solution on survival of *E. coli* strain 1303. 0.5 volumes of PBS or clarifying solution were added to 1 volume of raw or PBS spiked with 1 x 10^7 CFU ml^{-1} of *E. coli* strain 1303 and incubated for 15 min incubation on ice, before the samples were centrifuged and the bacterial count in the pellet was determined.

The different components of the clarifying solution were tested for their individual impact on bacterial survival (**Figure 7**). Accordingly, either EDTA or Triton X-100 was added to the raw milk and incubated for 15 min on ice.

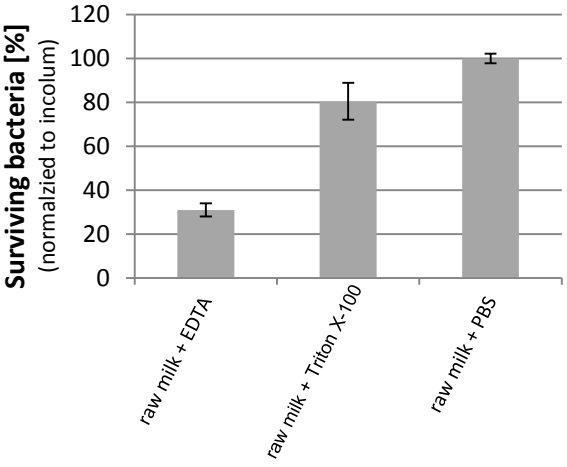

Figure 7: Survival of *E. coli* 1303 in raw milk upon addition of individual components of the clarifying solution. 0.5 volumes of EDTA (0.25 M) or Triton X-100 (0.5 % v/v) were added to 1 volume of raw milk spiked with 1 x 10^7 CFU ml^{-1} of *E. coli* strain 1303 and incubated for 15 min incubation on ice, before the samples were centrifuged and the bacterial count in the pellet was determined.

It turned out, that upon addition of EDTA (0.25 M) bacterial survival decreased by 70 %, whereas only 20 % of the bacteria were killed by addition of Triton X-100 (0.5 % v/v). The addition of EDTA most likely interfered with the stability of the Gram-negative cell envelope and thus resulted in increased bacterial killing. Even the use of clarifying solutions with decreased concentrations of EDTA and Triton X-100 resulted in significant reduction of bacterial counts in raw milk. Consequently, clarifying solution was not further used for bacterial isolation from raw milk.

V.2.2. Milk whey

It was assumed that for the analysis of *E. coli* isolates from mastitis and bovine faeces as well as for the identification of putative virulence genes the same methodologies should be applied that were used to investigate the virulence of other *E. coli* pathotypes (Shpigel et al., 2008). Unfortunately, growth or incubation of bacteria in raw milk resulted in irreproducible data, and hampered bacterial isolation and purification of sufficient amounts of pure bacterial RNA. Lacking methods to harvest bacteria from raw milk without a considerable loss of bacteria, we looked for a test system which approximately corresponds to the infectiologically relevant conditions provided by raw milk. Milk whey, defined as the fluid portion of milk remaining after removal of cell debris, casein and fat, turned out to fulfill the criteria of mimicking the infectiologically relevant conditions provided by raw milk. One exception is, that milk whey lacks the cellular components, e.g. somatic cells found in raw milk. Nevertheless, milk whey still contains defensins. As part of the host defense, defensins are released by cells of the innate immune system and assist in killing phagocytized bacteria. Usually, they are released by neutrophil granulocytes as well as by mucosal and epithelial cells when induced upon infection. Some are, however, constantly present in milk (Isobe, Morimoto, et al., 2009; Ganz, 2003). The replacement of raw milk by milk whey as a growth medium allowed us to assess various phenotypic and transcriptomic properties of *E. coli* isolates from bovine mastitis under conditions which mimic growth in raw milk, but circumvent the problems caused by fat, casein or somatic cells.

V.3. Phenotypic properties of *E. coli* bovine mastitis isolates

Virulence-associated traits as well as fitness traits of selected bovine mastitis or faecal *E. coli* strains were analyzed and identified in comparative phenotypic assays. For this purpose, we focused on comprehensive analyses at conditions either mimicking initial pathogenesis or conditions that *E. coli* mastitis isolates should encounter while entering the udder.

V.3.1. *E. coli* fitness in milk whey

For mastitis *E. coli* strains it is important to colonize the mammary gland. It has been suggested that bacterial multiplication is of high importance for the pathogenesis of *E. coli* bovine mastitis isolates. Rapid metabolical adaptation to mammary secretions subsequently leads to rapid increase of

bacteria numbers and elicits disease. Moreover, the severity of clinical mastitis and peak coliform counts in mammary secretions are positively correlated (Hogan and Smith, 2003). Before, however, mastitis causing *E. coli* are able to colonize milk ducts and mammary tissue, it is important to survive in milk. Bovine raw milk is known to exhibit antimicrobial activity and inhibits growth of *E. coli* which has been associated to the soluble, biologically active components in mammary secretions. These components mainly comprise **L**ingual **A**ntimicrobial **P**eptide (LAP) and other AMP/defensins, lysozyme, lactoferrin and lactoglobulin (Isobe, Nakamura, et al., 2009; Piccinini et al., 1999; Chaneton et al., 2008).

Blum and colleagues observed that *E. coli* mastitis isolates seemed to better multiply in the udder medium. They, however, used pasteurized milk to conduct their assay, so that antibacterial factors were probably neutralized (Blum et al., 2008).

We hypothesized that our native milk whey preserved the antimicrobial effects provided by the soluble factors of the innate immune system that are present in raw milk. In order to test the bactericidal activity of milk whey, we performed growth experiments and subsequently tested the survival of selected strains in different concentrations of milk whey.

Growth of individual mastitis *E. coli* isolates relative to bovine faecal *E. coli* isolates

The growth of *E. coli* isolates from bovine mastitis and bovine faeces in milk whey was comprehensively tested. For this purpose, we grew *E. coli* isolates aerobically in native milk whey at 37 °C while shaking. The initial inoculum was taken from stationary phase cultures grown in LB medium. Growth was monitored by quantification of CFUs on LB agar plates, because the translucent milk whey did not allow the measurement of the optical density. The resulting growth curves are shown in **Figure 8**. Each line represents a sigmoidal fit of the corresponding growth data. The growth curves of individual *E. coli* mastitis isolates were similar. In contrast, bovine faecal *E. coli* isolates differed in their ability to grow in milk whey and could be divided into two groups based on their growth characteristics. Although comparable inoculi (1 x 10^8 bacteria) have been used, two groups of faecal strains could be distinguished based on their survival in raw milk whey, which differed by two orders of magnitude.

The specific strain designation of the group 2 faecal isolates is indicated by red digits. The faecal isolates 2285, 2305, 2308 and 2340 exhibited substantially reduced bacterial numbers, which were by two orders of magnitude lower than that of group 1 faecal or mastitis isolates. Faecal isolate 2299 was also classified as group 2, because it visually showed the same growth characteristic, although its colony counts were in between the two groups for the first two hours.

Together, the fitness of group 2 faecal isolates in milk whey was reduced relative to that of group 1 faecal isolates, which behaved like mastitis isolates. Selected isolates from the group 2 faecal isolates have been introduced in other phenotypic assays and are therein designated in particular.

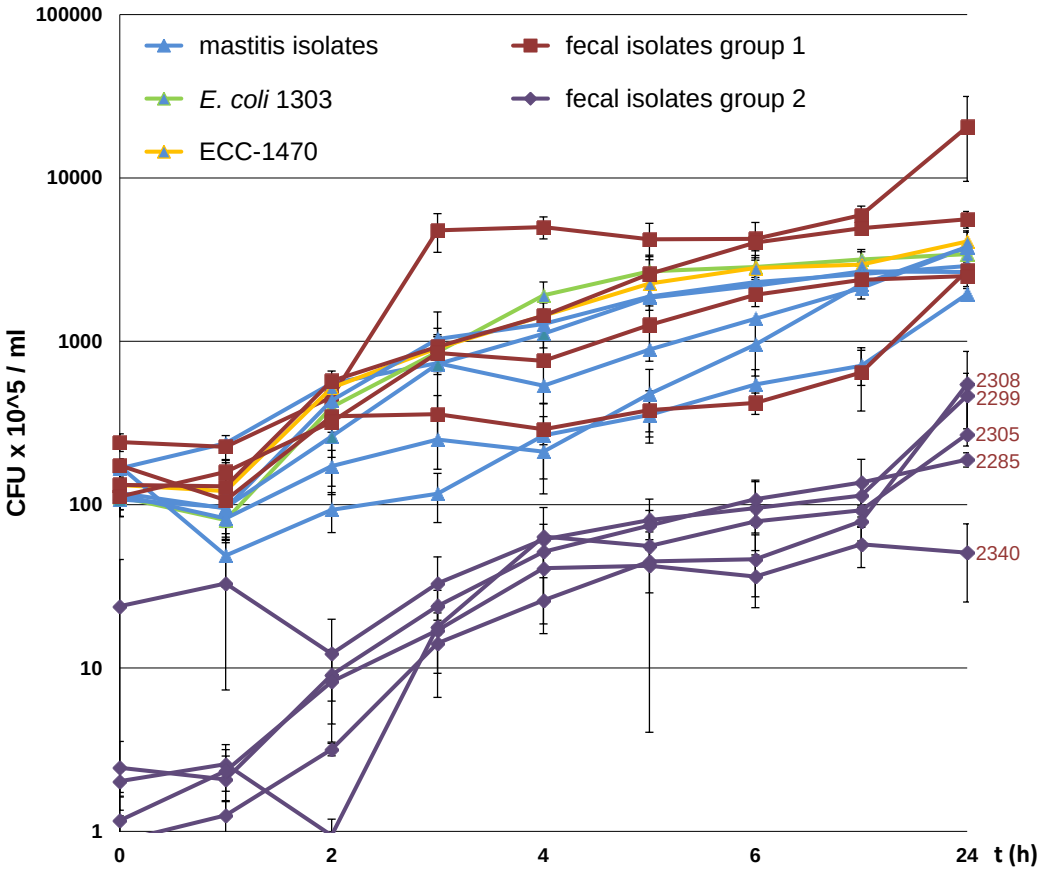

Figure 8: Survival of individual mastitis *E. coli* or bovine faecal *E. coli* isolates in milk whey. Growth of different mastitis and faecal *E. coli* isolates was compared in milk whey upon incubation at 37 °C for up to 24 h by quantification of CFUs on LB agar plates. Data show CFU averages of at least three independent experiments with error bars representing the SD of CFUs for each time point. Error bars very close to their correspondent data point might not be visible. Red digits indicate the designation of selected faecal isolates.

Sensitivity of selected bovine faecal *E. coli* isolates to milk whey relative to *E. coli* 1303

The survival properties in milk whey of the group 2 faecal isolates 2285, 2305, 2308 and 2340 were compared with those of mastitis *E. coli* strain 1303. For this purpose, we incubated the *E. coli* isolates aerobically in native milk whey at 37 °C without shaking for 10 minutes. Different ratios of native milk whey and LB medium (v/v) were used. The batches were inoculated as described before and the numbers of surviving bacteria were determined by CFU quantification on LB agar plates. The resulting survival characteristics are shown in **Figure 9**. The survival characteristics of the group 2 faecal isolates 2285, 2305, 2308 and 2340 were similar. They showed decreased survival abilities up to one order of magnitude, depending on increasing milk whey concentration.

In contrast mastitis *E. coli* strain 1303 did not differ in its ability to survive in milk whey and showed constant CFU counts when growing in up to 50 % milk whey. Regarding the short incubation time and the proportions of LB to milk whey, it is less likely that limited nutrients or iron shortage have

negatively influenced bacterial survival as both is sufficiently provided during the 10 minutes of incubation.

We conclude that the faecal isolates of group 2 are sensitive to milk whey relative to group 1 faecal isolates or mastitis *E. coli* strain 1303.

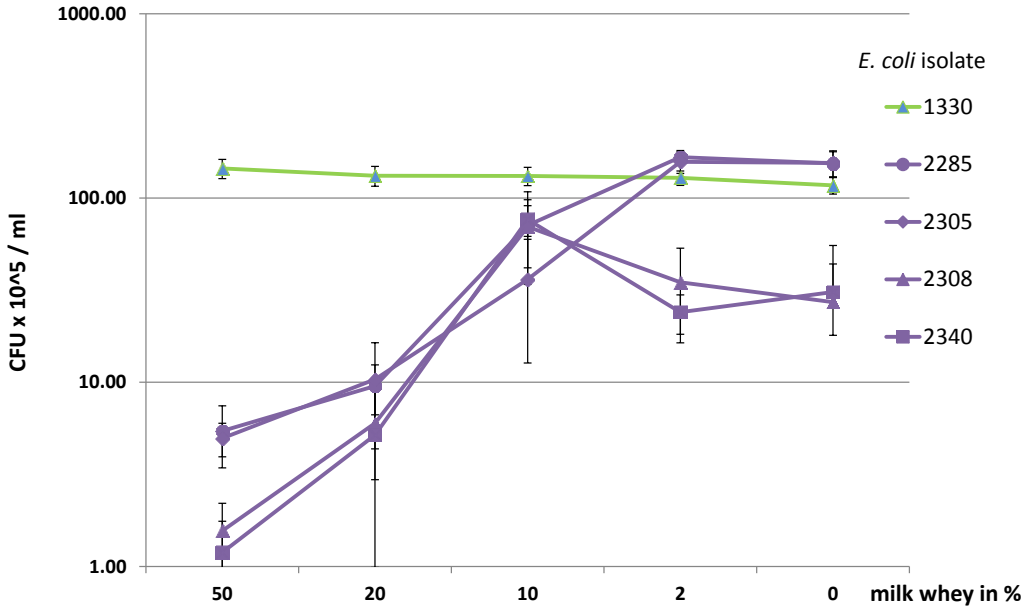

Figure 9: Sensitivity of selected bovine faecal *E. coli* isolates to milk whey relative to *E. coli* 1303. Survival of the different faecal *E. coli* isolates and mastitis *E. coli* strain 1303 was compared in milk whey upon incubation at 37 °C for 10 min by CFU quantification on LB agar plates. Data show CFU averages of at least three independent experiments with error bars representing the SD of CFU for each time point. Error bars very close to their correspondent data point might not be visible.

V.3.2. Lactose: A limiting nutrient in mammary secretions?

Life is strictly dependent on carbon and thus also *E. coli* has to utilize whatever carbon sources are available to fit its needs. In bovine milk, the major carbon source is lactose. Consequently, *E. coli* strains that are able to utilize lactose have a growth advantage in mammary secretions.

Growth and lactose utilization of *E. coli* isolates from bovine mastitis and bovine faeces were tested. For this purpose, we grew *E. coli* isolates aerobically in casamino acid- and glucose-deficient MM9 minimal medium at 37 °C. Lactose was used as the sole carbon source at a concentration of 0.2 %w/v. The initial inoculum was taken from stationary phase cultures of unmodified MM9 minimal medium containing casamino acids and glucose.

The resulting growth curves are shown in **Figure 10**. All strains were able to grow reasonably well, though the final cell density at OD_{600} differed from 0.24 to 0.57. Most cultures showed, however, no increase in their absorbance values in the first two hours of cultivation, probably due to adaptation

to the casamino acid- and glucose-deficient medium. After this lag-phase, growth proceeded normally. Interestingly, the group 2 faecal isolates 2299, 2340, 2308 and faecal isolate 2292 exhibited reduced fitness in comparison to the remaining faecal isolates, but did not differ from the several mastitis *E. coli* isolates.

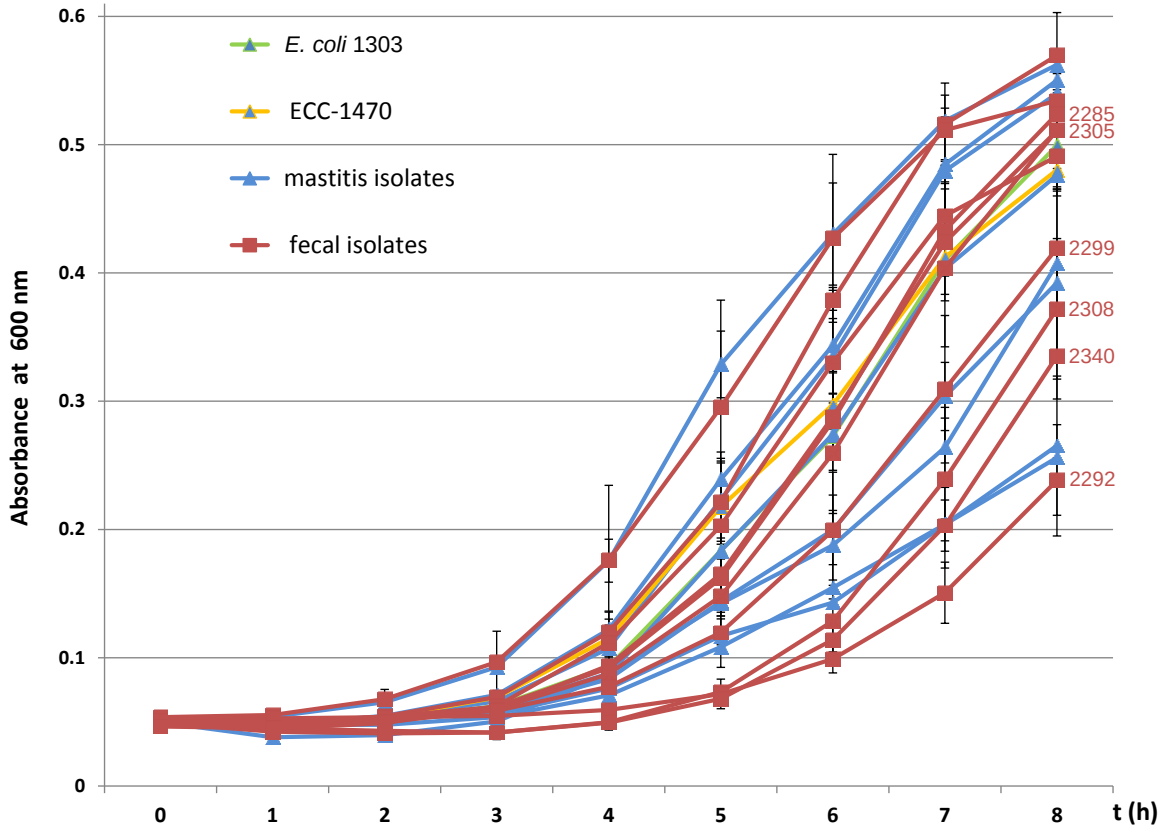

Figure 10: Growth of *E. coli* isolates on lactose. MM9 medium was supplemented with 0.2 % lactose as sole carbon source and strains were grown in aerobic batch cultures at 37 °C while shaking. Data points are shown as averages of at least three independent experiments with error bars representing the SD of absorbance values for each time point. Error bars very close to their correspondent data point might not be visible. Red digits indicate selected faecal isolates.

V.3.3. Influence of lactoferrin on *E. coli* isolates from mastitis and faeces

Lactoferrin is a major fraction of bovine milk and is known to bind iron, thus making it unavailable to bacteria (Clare and Swaisgood, 2000). Bacteriostatic activity of bovine lactoferrin *in vitro* was first observed by Reiter and Oram in 1967. Later, another study observed that lactoferrin had a bacteriostatic effect on all mastitis *E. coli* isolates tested, whereas only a few strains exhibited mild growth inhibition (Reiter and Oram, 1967; Rainard, 1986).

The growth of group 2 faecal isolates 2305 and 2308 relative to mastitis *E. coli* strains 1303 and ECC-1470 was comprehensively tested. For this purpose, we cultivated *E. coli* isolates aerobically in

LB medium with 2 mg ml^{-1} lactoferrin at 37 °C for 7 hours. The batches were inoculated from stationary phase cultures in LB medium with 1 x 10^8 bacteria. Each strain was tested in duplicate with and without lactoferrin and bacterial growth was determined by quantification of CFU on LB agar plates. CFU data are shown in **Figure 11**. Although the standard deviations were quite high, the presence of lactoferrin exhibited no significant bacteriostatic effect on neither group 2 faecal isolates 2305 and 2308 nor mastitis *E. coli* strains 1303 and ECC-1470.

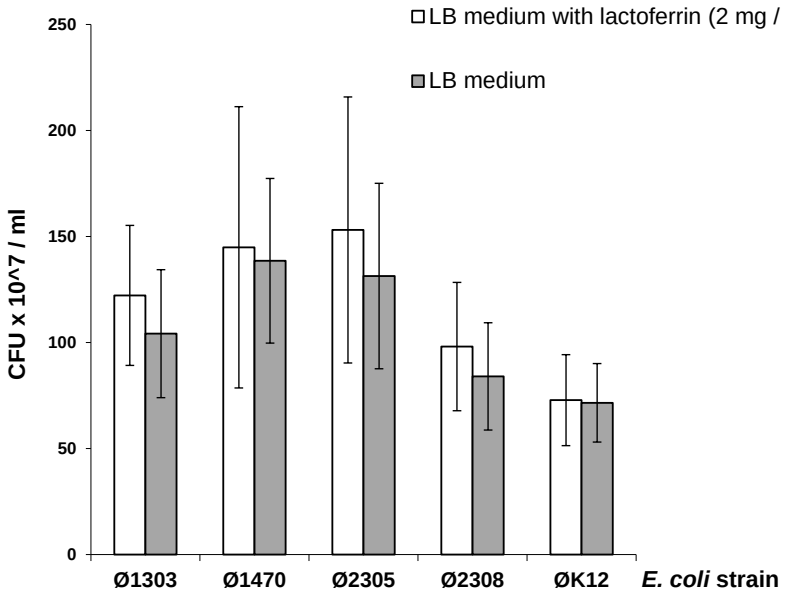

Figure 11: Influence of lactoferrin on growth and survival of mastitis *E. coli* isolates 1303 and ECC-1470 relative to milk whey-sensitive faecal *E. coli* isolates 2305 and 2308. Growth was compared in LB supplemented with a final concentration of 2 mg ml^{-1} lactoferrin upon incubation at 37 °C for 7 h. Data show CFU averages of two independent experiments with error bars representing the SD.

V.3.4. Influence of the lingual antimicrobial peptide (LAP) on survival of *E. coli* isolates from mastitis and faeces

The lingual antimicrobial peptide (LAP) is a member of the β-defensin family, which belongs to the group of antimicrobial peptides (AMP). Schonwetter and colleagues reported LAP expression in mammary epithelium in response to mastitis suggesting that LAP plays a role in the innate immune response to mastitis. Later, this was corroborated by the presence of functional LAP in bovine milk providing antimicrobial activity against *E. coli* (Schonwetter et al., 1995; Isobe, Nakamura, et al., 2009).). In return bacteria have evolved different mechanisms to resist the defensin-dependent killing (Peschel and Sahl, 2006). For mastitis *E. coli* it is important to resist to LAP as a functional antimicrobial component of milk. We, therefore, hypothesized that *E. coli* growing in milk whey might also express defensin-resisting mechanisms and are less susceptible to LAP. In order to test this

hypothesis we comprehensively investigated the fitness and survival capabilities of individual mastitis *E. coli* isolates relative to bovine faecal *E. coli* isolates to LAP. *E. coli* isolates were incubated aerobically in LB medium with different concentrations of LAP at 37 °C for 30 minutes. The batches were inoculated with stationary phase cultures in LB medium with 1 x 10^4 bacteria. Bacterial survival was determined by quantification of CFU on LB agar plates normalized to the initial inoculum. The relative survival of the isolates tested is shown in **Figure 12**.

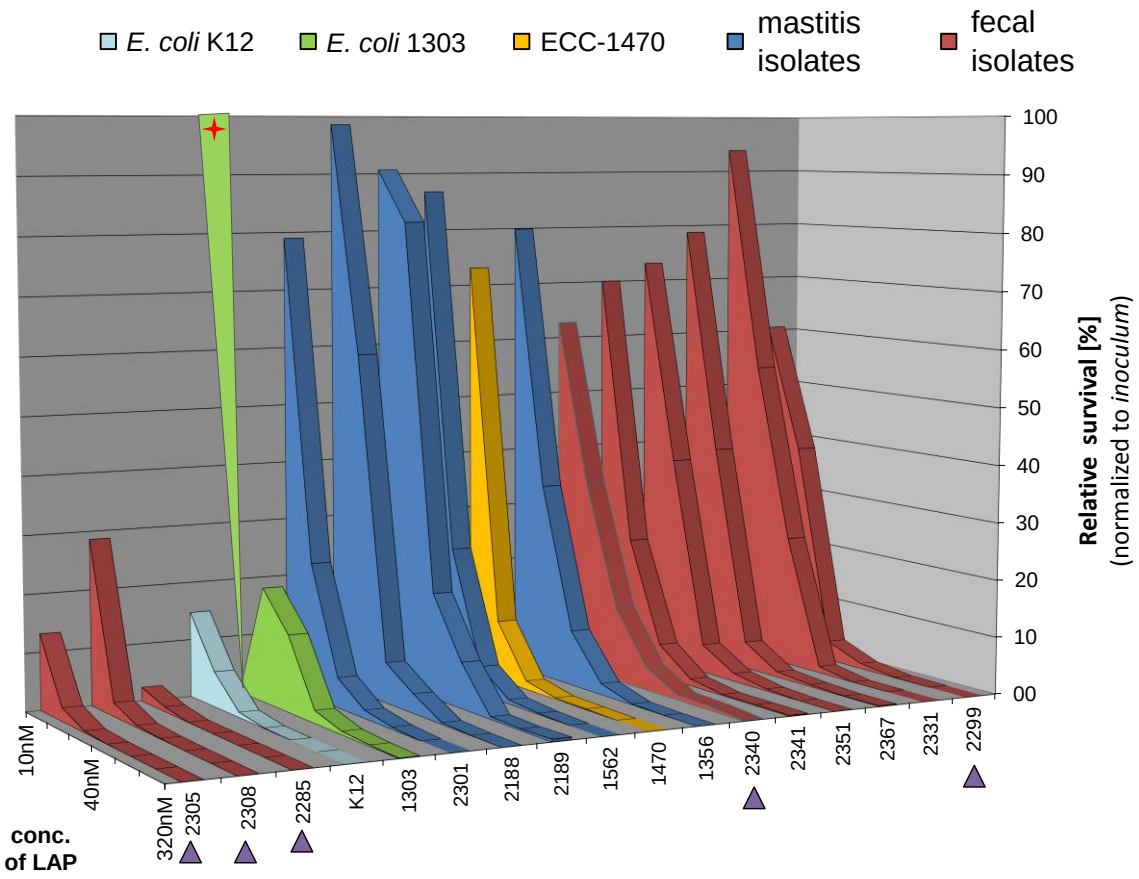

Figure 12: Sensitivity of individual mastitis *E. coli* isolates relative to bovine faecal *E. coli* isolates to LAP. Survival was compared in LB supplemented with different concentrations of lactoferrin upon incubation at 37 °C for 30 min by quantification of CFU on LB agar plates. Data show averages of relative survival of at least three independent experiments, normalized to the initial inoculum. Purple triangles indicate group 2 faecal isolates. The red star indicates data out of scale.

All isolates barely survived LAP concentrations higher than 80 nM. At concentrations below 20 nM LAP, relative survival of individual *E. coli* isolates is differing from 2.4 to 98.6 %. The colony counts of mastitis isolate *E. coli* 1303 exceeded the scale. Thus, *E. coli* 1303 exhibited the highest survival ability in presence of LAP. Additionally, all mastitis and most bovine faecal isolates showed substantial higher survival than group 2 faecal isolates 2305, 2308 and 2285 as well as *E. coli* K-12. In contrast, two out of the five group 2 faecal isolates, 2299 and 2340, survived equally relative to the mastitis

and the remaining faecal isolates. Together, we demonstrate that the presence of LAP resulted in significantly decreased bacterial numbers, though some faecal isolates were substantially more susceptible to LAP-mediated killing than all mastitis *E. coli* isolates and most faecal isolates tested.

V.3.5. What can we learn from the phenotypic assays?

E. coli mastitis strains are likely to originate from the faecal flora of the cow. There is evidence that their genetic and phenotypic variability is not reflected by *E. coli* environmental isolates of the cow, but might result from adaptation to an intramammary lifestyle (Bradley and Green, 2001; Dogan et al., 2006; Radostits et al., 2007; Blum et al., 2008). Metabolic adaptation to growth conditions in mammary secretions and the ability to rapidly multiply were suggested to be a prerequisite for *E. coli* bovine mastitis isolates. This is supported by a positive correlation between the severity of clinical mastitis and high bacterial counts in mammary secretions (Hogan and Smith, 2003). In the past, bactericidal activity and growth inhibition of *E. coli* by bovine raw milk have been associated to the activity of complement (Reiter and Brock, 1975). A broad diversity of biologically active components (e.g. macrophages, neutrophils, killer cells, immunoglobulins, complement, defensins, lysozyme, lactoferrin, cytokines) contribute to the antimicrobial effects provided by raw milk (Sordillo and Streicher, 2002). It is so far still difficult to experimentally determine their individual contribution to the overall antimicrobial and bacteriostatic effect, which is greater than the sum of the individual contributions due to their synergy.

Milk whey exhibits antimicrobial effects

Mastitis and faecal *E. coli* isolates exhibited similar growth characteristics when cultivated in LB, but exhibited remarkable differences when cultivated in milk whey. We initially hypothesized that our native milk whey exhibits, at least in part, the antimicrobial effects of raw milk. We demonstrated not only a bacteriostatic but also a bactericidal activity of milk whey, which cannot be related to the cellular antimicrobial components, because they are absent in milk whey. The preparation of milk whey removed fat, cell debris and even partially depleted casein which is also known to be an antimicrobial component (Malkoski et al., 2001). Especially the removal of milk fat avoids binding to and bacteriostatic effects that have been associated to milk fat globules (Sánchez-Juanes et al., 2009; Schroten et al., 1992) and thus may have biased colony counts and evaluation of antimicrobial effects by milk in previous evaluations.

Accordingly, in introducing milk whey to test the fitness and survival capability of *E. coli* isolates, we enabled comprehensive analyses which can specifically address virulence-associated traits important to resist the soluble biologically active components in mammary secretions. These components mainly comprise LAP and other AMP/defensins, lysozyme, lactoferrin and lactoglobulin (Isobe, Nakamura, et al., 2009; Piccinini et al., 1999; Chaneton et al., 2008).

E. coli* environmental isolates provide a reservoir of potential mastitic *E. coli

A previous study reported *E. coli* mastitis isolates multiply better in milk than most environmental *E. coli* isolates. The authors further identified a subset of environmental isolates with growth rates similar to those of mastitic isolates. However, they used pasteurized milk so that antibacterial factors were probably neutralized (Blum et al., 2008). Thus, a particular causality remains unclear.

We focused on the phenotypic characterization of mastitis *E. coli* isolates relative to isolates from bovine faeces of udder-healthy cows. We specifically addressed the question whether mastitis and environmental strains differ in their individual fitness traits upon selected conditions mimicking those in mammary secretions. On the one hand, we tested bacterial fitness in native milk whey, which we found to preserve antimicrobial effects in contrast to pasteurized milk. On the other hand, we respectively challenged selected isolates with iron shortage, lactose as sole carbon source and LAP as a main defensin of milk.

We clearly demonstrated that bovine faecal *E. coli* isolates represent a heterogeneous group of which some isolates showed a substantially reduced ability to survive in milk whey whereas others reflected the fitness of mastitic isolates that constitute a homogeneous group in that they show similar growth characteristics (**Figure 8**). This corroborates a previous report on growth of mastitis *E. coli* isolates (Blum et al., 2008) and extends the data to a greater number of isolates. In spite of what may appear at first glance to simply reinforce the findings of Blum et al., revealed a different outcome and added interesting aspects regarding the causality of reduced fitness.

Impact of lactoferrin

Lactoferrin is a predisposing antimicrobial protein of the soluble mammary gland defense components reported to increase in concentration in response to endotoxin (Schmitz, 2004; Sordillo et al., 1987). Due to its frequently observed growth inhibitory effect, which was suggested to be the main function of lactoferrin in previous studies (Chaneton et al., 2008; Chaneton et al., 2011; Rainard, 1986), we tested the susceptibility to lactoferrin on individual *E. coli* faecal isolates which we observed to exhibit reduced fitness in milk whey. Our data could neither proof nor deny this suggestion. The addition of lactoferrin to milk whey showed no significant bacteriostatic effect on either group, the faecal isolates of diminished fitness in milk whey or mastitis *E. coli* strain 1303 and ECC-1470 (**Figure 11**). We referred this to rather high standard deviations what obviously compromised unambiguous monitoring. Furthermore, it can be suggested that either LB is not a suitable laboratory medium to assess inhibitory effects of lactoferrin or that the utilized lactoferrin was corrupted. The first explanation would be supported by observations from Rainard and colleagues, who already emphasized the importance of the growth medium used in the inhibition assay. Specifically, lactoferrin was not causing complete bacteriostasis when the growth medium, consisting of casamino acids in PBS, was supplemented with 10% (v/v) brain heart infusion broth,

even when added in rather high concentrations 1 mg ml^{-1} (Rainard, 1986). It is, nonetheless, remarkable that the tremendously high concentration of 2 mg ml^{-1} lactoferrin relative to 0.1 mg ml^{-1} used by Rainard (1986), had no significant or even a visible effect on bacterial growth (**Figure 11**). On one hand, this might indicate a corrupted enzymatic activity of the lactoferrin used in our assay. On the other hand, no iron shortage was indicated by the transcription profiles of *E. coli* isolates 1303 and ECC-1470 incubated in milk whey.

The particular contribution of lactoferrin to *E. coli* bacteriostasis in mammary secretions needs to be further characterized. We hypothesize that lactoferrin might be beneficial in ongoing mastitis, when the *E. coli* intracellular iron stocks are emptied. This might be tested in real-time PCRs on samples obtained from *E. coli* incubation in milk whey for at least two hours, since a previous study hypothesized that the initial lag time is needed for depletion of cellular iron (Martin and Imlay, 2011).

Low impact of lactoferrin during initial growth of E. coli

In the present study we aimed for the characterization of virulence-associated traits, which may enable mastitis isolates as well as a subset of bovine faecal *E. coli* to successfully colonize the mammary gland and cause disease. When entering the mammary gland, the initial few hours are supposed to be most important for the outcome of the disease in that they are critical for bacterial survival. In contrast, an increase of lactoferrin gene expression and accumulation of the lactoferrin concentration in milk is known to occur rather late (Schmitz, 2004; Chaneton et al., 2008), suggesting other main roles of lactoferrin than inhibition of bacterial growth. An early investigation reported that high citrate levels during lactation compensate for the bactericidal effect of lactoferrin in milk due to the fact that citrate chelates iron thus providing a possibility for the bacteria to take advantage of this iron. In dry-periods citrate concentrations decrease and lactoferrin may then be responsible for maintaining bacteriostasis (Smith and Schanbacher, 1977). Besides its bactericidal effect, lactoferrin confers other properties such as detoxification by sequestering LPS and prevention of pro-inflammatory pathway activation by TLR4, sepsis and tissue damage (Latorre et al., 2010; Legrand et al., 2004). However, from the mastitis-causing bacteria species *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis* and *Escherichia coli* the species *E. coli* has been reported to be most susceptible to iron shortage (Rainard, 1986). Since we have not been able to detect lactoferrin-mediated bacteriostasis on our strains, this remains to be further investigated. We, however, presume that one or more iron uptake systems might enable mastitis-associated *E. coli* to overcome the inhibitory properties of lactoferrin. Notably, several genes associated with iron uptake could not be detected in *E. coli* 1303 and ECC-1470 (**Table 9**). Apart from that, other iron acquisition systems, such as enterobactin, seem to be conserved as it is commonly expressed by Gram-negative bacteria isolated from involuted mammary glands during the dry period. It has been shown that growth of *E. coli* was inhibited in mammary secretions by blocking iron uptake with antibodies

specific for the enterobactin receptor (Lin et al., 1999) indicating the importance of iron uptake systems in the context of mastitis.

Lactose fermentation: Not the key factor for survival in milk whey

It was previously discussed that environmental isolates that exhibited reduced fitness in milk were either less resistant to the biologically active components present in milk, or that they were less able to utilize the nutrients available in it, or both (Blum et al., 2008). Due to the fact that Blum and colleagues utilized pasteurized milk for their growth assay, they concluded that it is unlikely that the biologically active components influence bacterial fitness. Hence they proposed that the environmental isolates that showed diminished fitness in milk were less well adapted to metabolize the available lactose in milk in contrast to glucose in nutrient broth. This might be further supported by previous studies, which reported lactose fermenters to reach much higher bacterial counts in milk and, moreover, a positive correlation of the growth of a mastitis-associated *E. coli* strain to the percentage of lactose available (Hogan and Smith, 2003; Kornalijnslijper et al., 2003).

In contrast our findings considerably differed from those mentioned before due to the fact that the ability to grow on lactose did not unambiguously divided faecal isolates into one group with reduced fitness and into a second one that comprises mastitis isolates (**Figure 10**). Instead, faecal isolates with reduced fitness in milk whey did not differ from mastitis isolates regarding the distribution of the phenotypic ability to utilize lactose, though the overall growth performance on lactose greatly differed among all isolates tested (**Figure 10**). Especially, the faecal isolates 2285 and 2305 grew very well with lactose as sole carbon source, but showed reduced fitness in milk whey. This indicates that there is no direct correlation between multiplication in milk (whey) and a reduced ability to utilize lactose as it was suggested by Blum et al. Our finding is further supported by the fact that the general ability to use lactose seems to be a prevalent trait of *E. coli* as reported for disease-associated isolates as well as for faecal isolates of avian origin (Rodriguez-Siek et al., 2005). Furthermore, lactose is not the sole carbon source in bovine milk, which contains several oligosaccharides comprising galactose and glucose, which might be utilized by bacteria (Gopal and Gill, 2000). It is nevertheless of little doubt that especially mastitis *E. coli* which are adapted to an intramammary lifestyle, take advantage from using lactose as major carbon source **Figure 10**. At least, our finding that the faecal isolates were sensitive even to small portions of milk whey upon short incubation (**Figure 9**) indicated antimicrobial effects rather than nutrient shortage. Altogether, we report here that the ability to utilize lactose might at least promote fitness in milk whey, but is likely to be less important for successful mastitis than initially thought.

Reduced bacterial fitness related to LAP

Previous studies of *E. coli* growth inhibition in the mammary gland or mammary secretions focused on specific host defense mechanisms on the cellular level, but also suggested the action of complement, lactoferrin and lysozyme (Rainard and Riollet, 2006; Paape et al., 2003; Sordillo and Streicher, 2002). Analysis of bacterial growth depending on the availability of lactose and iron suggested nutrient shortage to be a bottle neck for *E. coli* multiplication (Chaneton et al., 2008; Blum et al., 2008; Rainard, 1986). The impact of antimicrobial properties present in mammary secretions is nonetheless questionable in that they provide an indispensable barrier effect to bacterial multiplication under physiological conditions.

E. coli mastitis isolates and most faecal isolates survive physiological LAP concentrations

In the present study, we focused on the specific antimicrobial effects provided by physiological concentrations of LAP. The physiological concentrations of LAP in milk have been recently determined to be about 17 nM under udder-healthy conditions and roughly 50 nM when the udder is infected with *E. coli*. Furthermore, a direct correlation between LAP concentration and SSC was shown (Kawai et al., 2013). Given the fact that the milk whey, which was utilized in our growth assays, derived from an udder-healthy cow we anticipate a similar concentration of LAP. This is in accordance with our observation that isolates able to survive LAP concentrations of 20 nM also performed well in milk whey. This suggests that fitness of *E. coli* isolates in raw milk is related to the ability to resist LAP.

LAP is a prominent β-defensins present in mammary secretions and its expression in mammary epithelial tissue is up-regulated during mastitis (Swanson et al., 2004). We demonstrated that the presence of LAP significantly decreased bacterial numbers (**Figure 12**). Furthermore, individual faecal isolates, which were also less fit in milk whey (**Figure 9**), were substantially more susceptible to killing by LAP than all mastitis *E. coli* isolates and most faecal isolates (**Figure 12**). This supports our hypothesis that LAP and other antimicrobial peptides are the main bactericidal agents in milk whey.

Adaption to an intramammary lifestyle requires resistance to LAP

Likewise other β-defensins, LAP is assumed to function by binding to the cell membrane, followed by the formation of pore-like membrane defects, which promote efflux of essential ions and nutrients (Ganz, 2003; Peschel and Sahl, 2006). *E. coli*, which are about to colonize a new niche like the mammary gland have to adapt not only to metabolic changes but also have to counteract defensins. Many bacteria evolved efficient countermeasures to confer increased resistance indicating the evolutionary pressure to colonize and infect their hosts. This might also include membrane-associated molecular mechanism to sense defensins and initiate transcription to express, e.g. extracellular proteases. The recognition of and resistance to host defensins is of high importance and

several studies correlated bacterial colonization to these particular traits. Specifically, defensin-susceptible mutants of Gram-positive bacteria have been attenuated in different infection models (Nizet et al., 2001; Kristian et al., 2003; Weidenmaier et al., 2005). Similarly, defensin-susceptible *Salmonella* mutants were attenuated in two independent studies that reported about the *pmr* locus, which is necessary for LPS modification in defensin resistance, as well as PhoP/Q two component system (Gunn et al., 2000; Guina et al., 2000). PhoQ is a sensor kinase important for the pathogenesis of a number of Gram-negative bacterial species and via its cognate response regulator PhoP it constitutes a signal-transduction cascade that controls inducible resistance to host defensins (Bader et al., 2005). *E. coli* can develop resistance by modification of their O-antigens by adding positively charged moieties, thus preventing the electrostatic interaction of defensins with their surfaces. Furthermore, extracellular proteases such as OmpT are known to degrade α-helical AMPs thereby providing resistance to defensins (Thomassin et al., 2012; Stumpe et al., 1998). Moreover, biofilm, which is a common virulence-associated trait of many ExPEC, is also known to provide a generally protective coverage to antimicrobial effects including those of defensins (Otto, 2006).

By identification of *E. coli* bovine faecal isolates, which are resistant to particular concentrations of LAP (**Figure 12**), we provided evidence for a subset of *E. coli* strains that could be phenotypically more adapted than other environmental strains to an intramammary lifestyle.

E. coli isolates conferring LAP resistance do not unambiguously thrive in milk whey

Interestingly, two out of the five designated group two faecal isolates with reduced fitness in milk whey (**Figure 9**), exhibited equal survival ability to LAP likewise the mastitis *E. coli* isolates and the remaining faecal isolates (**Figure 12**). Hence, their reduced fitness in milk whey must rely on other factors than LAP resistance. A limited ability to utilize lactose was excluded due to the isolates´ reasonably well growth in the lactose utilization assay (**Figure 10**). Furthermore, growth inhibition by the bactericidal lactoglobulin can be excluded, because it was shown to inhibit the growth of *S. aureus* and *S. uberis*, but not of *E. coli* (Chaneton et al., 2011). It should also be noted that resistance to defensins might not be a general molecular mechanism, but can be highly specific to the particular defensin. We, therefore, suggest that the reduced fitness of faecal isolates unsusceptible to LAP resulted from susceptibility to other milk whey components, which have not been assessed such as other defensins present in milk. The contribution of lysozyme to the antimicrobial effect of mammary secretions was not evaluated to date whereas its concentration in bovine milk is extremely low (130 ng ml^{-1}) when compared to human milk (10 mg ml^{-1}) (Hettinga et al., 2011). In contrast, the number of discovered β-defensins is still increasing and to date the β-defensin family present in mammary secretions comprises not only LAP, but bovine neutrophil β-defensin, tracheal antimicrobial peptide, enteric β-defensin and bovine β-defensin (Kawai et al., 2013).

Considerations

It is an ongoing discussion whether a specific set of *E. coli* constitutes a mammary pathogenic group of *E. coli* or if any *E. coli* is able to cause mastitis when the determining factors are relying on the host. Our data could neither proof nor deny either one of both suggestions. On the one hand, *in vitro* exposure of bacteria to slowly increasing defensin concentrations over several hundred generations has been shown to result in reversible physiological adaptation and/or spontaneous, inheritable resistance to the peptide used (Peschel and Sahl, 2006). On the other hand, we can only assume how fast such adaptations might occur *in vivo* and how efficient and specific these adaptations might be when taking into account that all mentioned defensins present in milk create simultaneous selective pressure. Previous studies reported a significant increase of LAP concentrations in response to LPS as well to *E. coli*, when injected into the mammary gland, which remained at high levels for two days (Isobe et al., 2009a and 2009b). A comparison of the growth curve regression slopes showed no evidence that mastitis *E. coli* isolates were multiplying significantly faster relative to faecal *E. coli* isolates in milk whey. This suggests that adaptation to mammary secretions is rather inherent than an adaption "on the run". We propose further comprehensive investigations of mastitic *E. coli* and faecal isolates to test whether biofilm formation or susceptibility to further defensins are important for growth in mammary secretions.

V.3.6. Adhesion and invasion of selected mastitis *E. coli* strains *in vitro*

The pathogenesis of *E. coli* mastitis is assumed to resemble the pathogenesis of urinary tract infections in that the infection is also ascending (Kaipainen et al., 2002; Ghanbarpour and Oswald, 2010). The *E. coli* isolates causing these infections are commonly classified as ExPEC, which often can adhere, invade and persist in host epithelia (e.g. UPEC and MNEC) (Kaper et al., 2004).

In this study, the cell adhesion and invasion potential of model mastitis isolates, strain 1303 (acute mastitis isolate) and ECC-1470 (persistent mastitis isolate), were investigated. Additionally, we tested for effects of milk whey on *E. coli* cell adhesion. We expected results, which might be correlated with data from transcriptional analyses during cocultivation of *E. coli* mastitis isolates in the presence of mammary gland epithelial cells (section V.5). For this purpose we performed a cell adhesion assay and a gentamycin protection assay in parallel, as described in section IV.5.2 and IV.5.3. To examine effects of milk whey on bacterial adhesion to eukaryotic cells, we performed a second adhesion assay in parallel where the cell culture medium was in part replaced by milk whey. Different concentrations of raw milk whey were tested before and no inhibitory effects on MAC-T cells immortalized mammary gland epithelial cells was observed when cultivated in 60 % milk whey : 40 % cell culture media (v/v) (**Figure 13**).

No cytotoxicity was detected with the bacterial multiplicities of infection (MOI) used in the adhesion and invasion assays. The strains *E. coli* MG1655 Δ*fim*Δ*flu* and *S. typhimurium* SL1344 were used as

controls. All strains were allowed to adhere to MAC-T cells for 2h. The bacterial numbers (colony formation units) observed were plotted in relation to the initial inoculum (**Figure 14**).

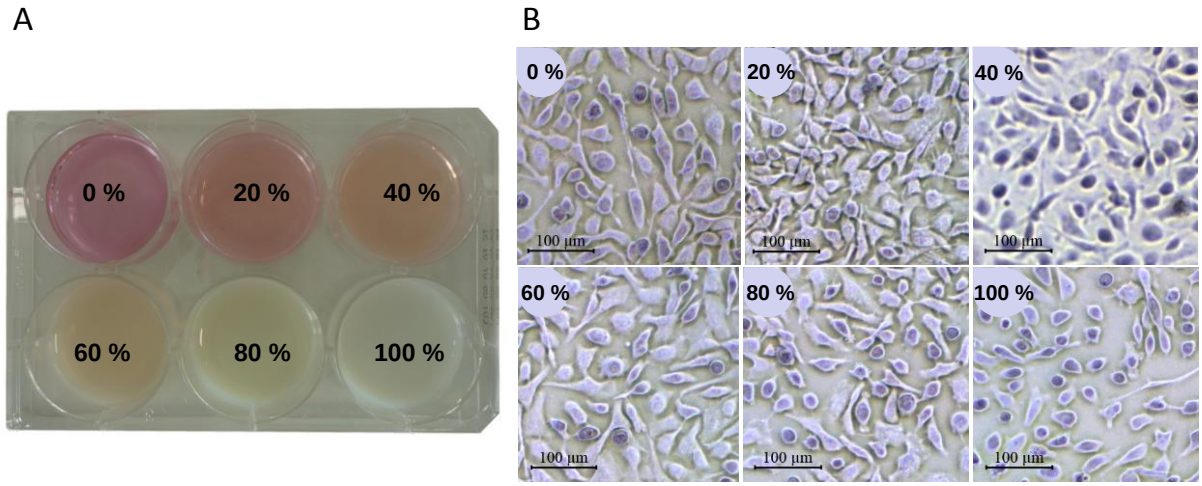

Figure 13: Mammary gland epithelial cells in milk whey. The MAC-T cells were incubated in different ratios of sterile milk whey and cell culture media (DMEM high Glucose + 5 % FCS). The percentage of milk whey used is indicated in the individual wells (A) and their morphology was examined after20 h of incubation (B). MAC-T cells incubated in the presence of > 60 % milk occurred to be hypotonic.

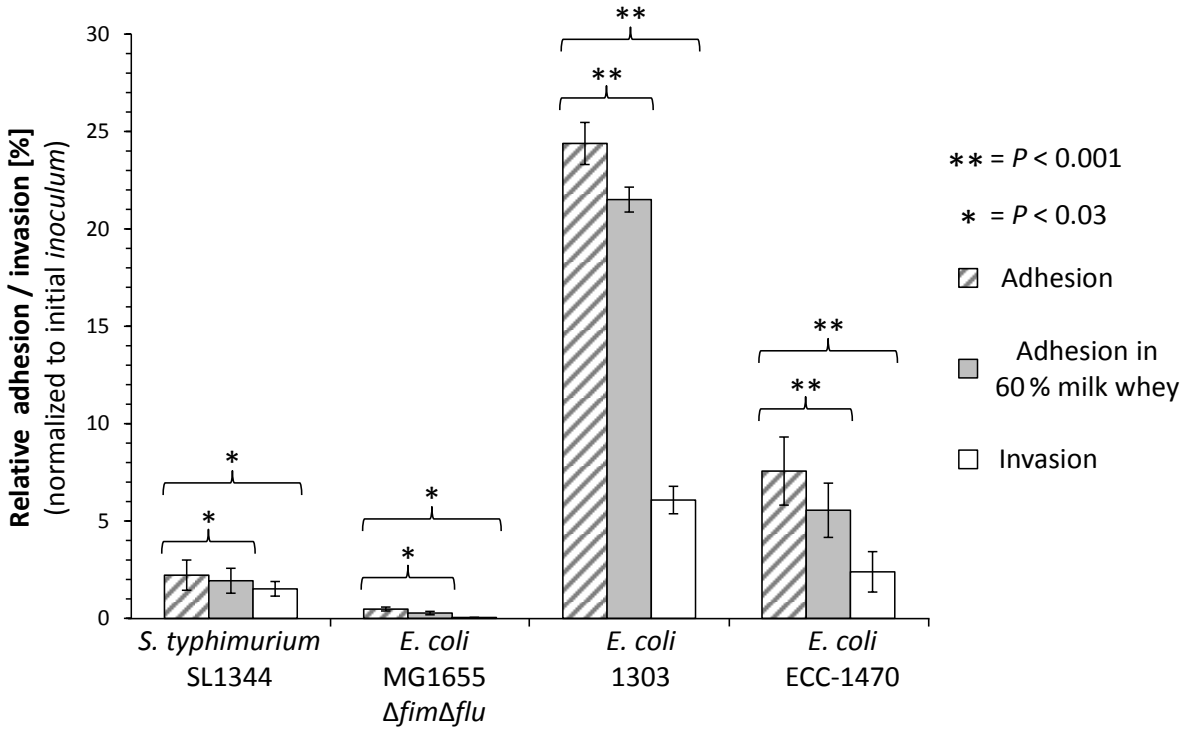

Figure 14: Adhesion and invasion of *E. coli* mastitis isolates to MAC-T mammary gland epithelial cells. The strains were allowed to adhere to or invade into MAC-T immortalized mammary gland epithelial cells for 2 h. The different assays were conducted in parallel. Data represent relative (i) adhesion (hatched) or (ii) adhesion in 60 % milk whey (grey) or iii) invasion (white), normalized respective the initial inoculum. Data are the mean ± standard deviation (SD) of at least three independent experiments in triplicate wells. Asterisks indicate a statistical significance (*, $P < 0.03$; **, $P < 0.001$).

Adhesion to and invasion into bovine mammary gland epithelial cells *in vitro*

The data displayed in **Figure 14** show a significantly stronger interaction of *E. coli* strain 1303 and ECC-1470 with immortalized bovine mammary gland epithelial cells compared to the control strains *E. coli* MG1655 Δ*fim*Δ*flu* and *S. typhimurium* 1344. The comparison of individual strains resulted in a wide range of differences between strains and either adhesion or invasion. The negative control *E. coli* K-12 strain MG1655 Δ*fim*Δ*flu* showed less than 0.5 % adhesion and no significant internalization, which was expected. This strain lacks the Ag43 coding gene *flu* and the type 1 fimbriae coding genes *fimB-H* avoiding expression of factors that could contribute to bacterial biofilm formation, autoaggregation, and/or adherence (Reidl et al., 2009). The control strain *S. typhimurium* 1344 showed a merely low adhesion rate of 2.22 %, which is in accordance with adhesion levels reported for *S. typhimurium* with respect to the utilized cell model (Gagnon et al., 2013). The acute mastitis isolate *E. coli* 1303 showed a three times higher adhesion rate and was also 2.5 times more invasive than the persistent mastitis isolate ECC-1470. Interestingly, the presence of 60 % milk whey significantly reduced the adhesion to the mammary gland epithelial cells by 11.8 % for *E. coli* 1303 and 26.6 % for ECC-1470.

Different adhesion and invasion properties of *E. coli* strain 1303 and ECC-1470

The adhesion of a clinical mastitis isolate (*E. coli* strain P4) to epithelial cells, from the teat and lactiferous sinuses of the udder, was first observed in 1978 by Harper et al.. At this time, the adhesion was demonstrated to be fimbria-dependent due to mannose inhibition. Another study observed that *E. coli* clinical mastitis isolates adhered to and invaded into cultured bovine mammary gland epithelial cells. Moreover, isolates from persistent intramammary infections with recurrent cases of clinical mastitis invaded comparatively faster and in larger numbers than the strains from single cases (Döpfer et al., 2000). This was corroborated by another study, which showed that persistent *E. coli* mastitis isolates, amongst others ECC-1470, adhered exhibited similar adhesion rates, but invaded more efficiently into mammary gland epithelial cells than transient *E. coli* mastitis isolates (Dogan et al., 2006).

The results of the present study are in contradiction to this general message. We clearly demonstrated a substantially higher adherence and invasiveness of *E. coli* strain 1303, which has been classified as a transient mastitis strain in comparison to the persistent mastitis strain ECC-1470 (**Figure 14**). Strain ECC-1470 exhibited a significantly higher invasion rate than *E. coli* 1303 in relation to their respective adhesion capacity (ANCOVA; $F_{1,14}$= 291.69, $P < 0.001$, where the initial inoculum was included as a covariate). It can be hypothesized that this difference might even increase over time and confers a faster internalization of *E. coli* strain ECC-1470 into mammary gland epithelial cells than *E. coli* strain 1303 what might increase its persistence potential: In particular, the intracellular lifestyle and evasion from the host´s immune response. On the other hand, the transient mastitis

E. coli isolate 1303 has been shown to adhere and invade mammary gland epithelial cells in remarkably greater numbers than persistent mastitis isolate ECC-1470 (**Figure 14**). This leads to the assumption that *E. coli* strain 1303 expresses traits contributing to adherence and invasion different from ECC-1470. Whether these traits are more efficient *per se* or whether they confer enhanced adhesion due to synergistic effects required further verification.

For mastitis *E. coli* strains it is important to colonize the mammary gland including the stratified epithelia lining the luminal walls of the teat canal and cistern, gland cistern, milk tubules and the specialized alveolar epithelium. This requires factors contributing to cell adhesion and invasion promoting virulence. Dogan et al. suggested that the phylogenetic group and the presence of long polar fimbriae (LpfA) influence the epithelial invasion by persistant *E. coli* mastitis isolates (Dogan et al., 2012). Interestingly, the corresponding gene *lpfA* is absent in transient *E. coli* mastitis strain 1303 (phylogroup A), but present in persistent mastitis strain ECC-1470 (phylogroup B1) (**Table 9**). Its expression was not deregulated in the presence of bovine mammary epithelial cells relative to their absence (see section V.5). This suggests that the remarkably higher adhesion rate of *E. coli* 1303 is likely to be due to other factors since *lpfA* was related to persistent *E. coli* mastitis isolates such as ECC-1470, which exhibited less adhesion (**Figure 14**). Nevertheless, both strains carry 37 (*E. coli* 1303) to 52 (ECC-1470) genes within their genomes, which encode for fimbrial adhesins or predicted fimbrial-like adhesins.

It is, however, questionable whether cell adhesion occurs *in vivo* in the lactating cow, because several studies reported a reduction of adherence of *E. coli* but also of *Salmonella* spp. by whole milk, milk fat globules and their components (Sánchez-Juanes et al., 2009; Guri et al., 2012; Schroten et al., 1992; Atroshi et al., 1983; Harper et al., 1978).

Milk whey negatively influences bacterial adhesion to mammary gland epithelial cells

The cocultivation of *E. coli* mastitis isolates and mammary gland epithelial cells in presence of milk whey was originally considered to allow comprehensive analyses in a setting that, at least partially, mimics the environment during initial mastitis pathogenesis. In order to examine effects of milk whey on cell adhesion, we performed an adhesion assay where the pure cell culture medium was replaced by 60 % milk whey.

Our data clearly revealed a significantly reduced ability of all bacterial strains tested to adhere to cultured mammary gland epithelial cells (**Figure 14**). This effect was observed to be 2 times stronger for strain ECC-1470 than for *E. coli* 1303 (ANCOVA; $F_{1,14}$= 2391.32, $P < 0.001$). It can be presumed that the substantially weaker cell adhesion by *E. coli* ECC-1470 caused by milk whey, might also be responsible for this strain's more efficient invasion potential indicating its intracellular lifestyle. Due to our observations in the cell adhesion assay we may hypothesize that i) either milk whey inhibits bacterial adhesion to eukaryotic cells or ii) that the number of viable bacteria was decreased,

because of AMP interactions. This might subsequently reduce their overall adhesion rate (see also section V.3.1).

On the other hand, the latter hypothesis is unlikely due to the fact that mastitis isolate *E. coli* 1303 showed no decreased survival when incubated in up to 50 % milk whey (**Figure 9**). Moreover, the growth characteristics of both mastitis strains *E. coli* 1303 and ECC-1470 revealed equal fitness when grown in milk whey (**Figure 8**). We, therefore, presume that milk whey directly inhibits bacterial adhesion to eukaryotic cells by other mechanisms than antimicrobial ones. This is further corroborated by the fact that whole milk has been previously observed to inhibit the adhesion of *E. coli* to cells *in vitro* (Harper et al., 1978). The molecular mechanism of inhibition of fimbria-mediated *E. coli* cell adhesion is thought to be provided by milk fat-globule membrane components and glycosphingolipids (Sánchez-Juanes et al., 2009; Schroten et al., 1992; Atroshi et al., 1983). We might, however, highlight the fact that cell debris and milk globules were almost completely removed from raw milk during processing to milk whey (see section III.4.1) indicating that fat globules are unlikely to cause the effect we observed. It is, however, interesting that the inhibitory capacity of bacterial cell adhesion is suggested to depend on host species. For example, bovine, goat and human milk have been shown to markedly reduce bacterial binding and invasion relative to controls. Specifically, the fat globules derived from bovine milk were observed to provide weaker protective traits than those derived from goat or human (Guri et al., 2012; Tellez et al., 2012; Schroten et al., 1992).

Our results strongly suggest that milk properties conferring protection against bacterial adhesion might be also provided by other components in milk. The particular mechanism of the inhibition of *E. coli* adhesion to bovine mammary gland cells in milk whey remains unclear. Thus, an inhibitory effect provided by components other than milk fat-globule membrane components and glycosphingolipids might contribute to the overall inhibitory effect. Vice versa, particular *E. coli* strains might resist either one of the adhesion inhibitory traits and might thus outcompete strains, which are not resistant. It is, however, necessary to comprehensively investigate whether milk fat globule components are the only inhibitory agent in *E. coli* cell adhesion by milk whey while there is reason to doubt. We therefore suggest testing of different milk fractions, respectively, in comparative cell adhesion assays.

V.4. Autotransporter genes in mastitis isolates

A marked prevalence of many known virulence-associated genes of ExPEC and IPEC among *E. coli* mastitis isolates has not yet been published. Instead, *E. coli* mastitis isolates commonly lack known virulence markers (Blum and Leitner, 2013; Silva et al., 2013; Cheng et al., 2012; Dogan et al., 2012; Kerro Dego et al., 2012; Fernandes et al., 2011; Suojala et al., 2011; Ghanbarpour and Eric Oswald,

2010; Dyer et al., 2007; Wenz et al., 2006; Bean et al., 2004; Lehtolainen et al., 2003; Kaipainen et al., 2002; Wise et al., 2002; Lipman et al., 1995; Sanchez-Carlo et al., 1984).

In former studies autotransporter (AT) proteins were repeatedly considered virulence- associated factors (Ulett et al., 2007; Allsopp et al., 2010 and 2012; Totsika et al., 2012). The functional redundancy and wide distribution of many ATs among pathogenic and non-pathogenic *E. coli* indicates that ATs cannot generally be regarded as specific biomarkers and virulence factors (VFs) *per se* (Zude et al., 2014). Nonetheless, AT proteins promote colonization of intestinal and extraintestinal sites by ExPEC and thus AT expression can be considered advantageous in mastitis pathogenesis.

We, therefore, evaluated the prevalence of selected AT-encoding genes among *E. coli* isolates from bovine mastitis in order to elucidate whether a possible accumulation of AT genes might promote colonization of the udder. Furthermore, we extended this evaluation to include *E. coli* isolates from faeces of healthy cows as *E. coli* is considered an environmental pathogen of mastitis (Radostits et al., 2007; Bradley and Green, 2001). Based on differences between *E. coli* mastitis isolates and such from the environment of the cow, it has been suggested that clinical bovine mastitis *E. coli* isolates may form a subset of the environmental *E. coli* population. Moreover, mastitis isolates seemed to better multiply in the udder medium and to evade the host cellular innate immune response. Mastitis isolates were also anticipated to be genetically distinct from most environmental strains (Blum et al., 2008). Interestingly, the genetic distinction failed in a more recent study of the same working group (Blum and Leitner, 2013). However, we were interested in determining whether there is a difference in AT prevalence between mastitis and faecal isolates. For this purpose we performed a PCR-screening to screen the selected *E. coli* isolates (**Table 6**) for the presence of the AT genes *upaB*, *upaC*, *upaI* and *upaJ*. The primers were designed from specific nucleotide sequence sections (of the α-domain-encoding region of the respective AT genes,) with a low prevalence of repetitive sequences (**Table** 7).

Additionally, we tried to elucidate whether the prevalence of the examined ATs in mastitis isolates and isolates from bovine faeces would be similar in a strain collection comprising mainly human pathogenic isolates (amongst others various IPEC, UPEC, sepsis and faecal isolates; **Table 6**). Therefore, the strain collection of the Institute of Molecular Infection-Microbiology (IMIB) of the University of Würzburg was investigated as well which comprises different clinical isolates from various non-bovine sources.

Distribution of autotransporter genes among mastitis and bovine faecal isolates

The PCR screening of 218 mastitis isolates and 72 isolates from bovine faeces resulted in the data shown in **Table 10**. Of the 218 mastitis isolates, 65 (29.82 %) had at least one AT gene detected by PCR. The most common AT of mastitis, bovine faecal and non-bovine IMIB isolates was *upaB*, which was detected in 55 (25.23 %) of the mastitis isolates. All AT genes were either present alone or in

combination with each other. Although the mastitis, bovine faecal and non-bovine IMIB isolates were not of comparable number, the mastitis and bovine faecal isolates did significantly differ in the presence of the four AT genes. Between the isolates of the IMIB collection and the bovine faecal isolates, the AT genes *upaC*, *upaI* and *upaJ*, but not *upaB* significantly differed in their prevalence. Notably, the prevalence of *upaB* was exceptional in that it did not differ that much between these groups. In general, the prevalence of all four AT genes declined from the IMIB isolates to bovine faecal isolates and from bovine faecal isolates to mastitis isolates.

Table 10: Presence of selected autotransporter genes in bovine mastitis, bovine faecal and other non-bovine isolates

Number of tested isolates	***upaB***	***upaC***	***upaI***	***upaJ***
218 mastitis isolates	55 [25.23 %]	15 [6.88 %]	10 [4.59 %]	2 [0.92 %]
72 bovine faecal isolates	24 [33.33 %]	11 [15.28 %]	8 [11.11 %]	0 [0.00 %]
183 isolates of IMIB collection	65 [35.52 %]	51 [27.87 %]	51 [27.87 %]	8 [4.37 %]
Total isolates (473)	144 [30.44 %]	77 [16.28 %]	69 [14.59 %]	10 [2.11 %]

In order to compare these observations with data from an AT gene screening of a strain collection with emphasis on the AT distribution in the known major pathotypes, we took advantage of and re-illustrated data from another screening performed in parallel (**Table 11**). Notably, these data were achieved by an *in silico* analysis of publicly available complete genome datasets of a comparable number of pathogenic and non-pathogenic isolates. Our first observation was the significantly higher prevalence of all four AT genes among ExPEC isolates relative to IPEC and non-pathogenic isolates. Nevertheless, the presence of these AT genes was still much higher in comparison to the mastitis, bovine faecal and non-bovine isolates (**Table 10**). Of note, the prevalence of *upaB* was fairly the same in the IPEC and non-pathogenic isolates according to the complete genome sequence screening (Table 10) and the isolates from bovine faeces and non-bovine isolates of the IMIB collection screened by PCR (Table 9).

Table 11: Distribution of individual AT homologs in relation to pathotype (data from Zude et al., 2013)

Number of analyzed genomes	***upaB***	***upaC***	***upaI***	***upaJ***
9 ExPEC isolates	6 [66.66 %]	8 [88.88 %]	7 [77.77 %]	2 [22.22 %]
52 IPEC isolates	18 [34.62 %]	24 [46.15 %]	11 [21.15 %]	6 [11.54 %]
50 non-pathogenic isolates	18 [36.00 %]	28 [56.00 %]	13 [26.00 %]	2 [4.00 %]
Total isolates (111)	42 [37.84 %]	60 [54.05 %]	31 [27.93 %]	10 [9.00 %]

No crucial role of autotransporters in mastitis

The low prevalence of the selected AT genes *upaB*, *upaC*, *upaI* and *upaJ* among the 218 mastitis isolates suggests that they may not play a crucial role for the development of mastitis. Isolates from bovine faeces were investigated as well to find out whether mastitis and faecal isolates differ in the presence of virulence-associated genes. It has been suggested that mastitis strains originate from the faecal flora of the cow (Radostits et al., 2007). Moreover, the genetic as well as phenotypic variability of *E. coli* mastitis isolates seems not to be reflected by *E. coli* environmental isolates of the cow, but might be a product of adaptation to an intramammary lifestyle (Bradley and Green, 2001; Dogan et al., 2006; Blum et al., 2008). Our data could neither proof nor refute this suggestion.

On the one hand, the screened mastitis isolates revealed a significantly lower AT prevalence in comparison to the bovine faecal isolate as well as all the different sets of major *E. coli* pathotypes examined. This is in contradiction to the hypothesized accumulation of AT genes, in case of advantageous fitness provided by ATs. Hence, the colonization of the udder is likely to rely on other factors than ATs.

On the other hand, we do know that ATs often promote functions which are redundant to other AT functions (Zude et al., 2014). Furthermore, the total virulence potential of a pathogen is often greater than the sum of the individual contributions of virulence-associated traits, what is most likely due to, at least in part, their synergy. Thus, AT proteins might still improve the colonization of extraintestinal sites. Additionally, the relatively low frequencies of other virulence-associated factors should be considered, which often not exceeding 30 % prevalence of genes as reported in various studies of *E. coli* mastitis, (Blum and Leitner, 2013; Silva et al., 2013; Cheng et al., 2012; Dogan et al., 2012; Kerro Dego et al., 2012; Fernandes et al., 2011; Suojala et al., 2011; Ghanbarpour and Oswald, 2010; Dyer et al., 2007; Wenz et al., 2006; Lehtolainen et al., 2003; Kaipainen et al., 2002; Wise et al., 2002; Lipman et al., 1995; Sanchez-Carlo et al., 1984). It can be concluded that the 29.82 % AT prevalence among mastitis isolates may not be neglected, but anticipate ATs to take place in a much bigger set of factors that promote a successful colonization of the udder. Nevertheless, an application as biomarkers for a presumed MPEC pathotype cannot be proposed for either one of the four AT genes.

AT prevalence influenced by phylogeny rather than pathotype classification

The significantly lower prevalence of the three AT genes *upaC*, *upaI* and *upaJ*, but not *upaB*, among the isolate groups shown in **Table 10** in comparison to the isolate groups shown in **Table 11** seems to be inconsistent. Several studies try to explain this kind of discrepancy between studies with either different sampling techniques or different assessment methods (Blum and Leitner, 2013; Blum et al., 2008). In our case, the data set of the present work was achieved by PCR-screening (**Table 10**) whereas the data shown in **Table 11** were achieved by an *in silico* query. Nevertheless, both methods

proved to be reliable (Zude et al., 2014; Wells et al., 2009). In the present work, AT gene-specific primers were designed and repeatedly proven to be functional in PCR-screenings, leaving no doubt of the presence of AT genes as revealed in **Table 10**. Thus, we might exclude a marked influence by the two diverse assessment methods. We rather suggest an influence on AT prevalence caused by the strain selection and its implied phylogenetic diversity. This is supported by the general distribution of *E. coli* mastitis and bovine environmental isolates, mainly into the phylogeny groups A and B1 (Blum and Leitner, 2013; Ghanbarpour and Oswald, 2010). Another study even reported both persistent and transient isolates of *E. coli* of group A exclusively (Dogan et al., 2006). It is common knowledge nowadays that group A comprises mostly commensal *E. coli* while group B1 constitutes an assortment of different pathotypes and commensals. Both phylogeny groups, A and B1, are sister taxa of the youngest lineages in *E. coli* phylogeny whereas the earlier emerged group B2 comprises many ExPEC strains (Leimbach et al., 2013; Clermont et al., 2000). Interestingly, the study by Zude et al. determined the highest AT prevalence per strain in isolates of phylogroup B2, followed by B1. Moreover, it has been shown that AT distribution correlates rather with phylogenetic lineages than with pathotypes (Zude et al., 2014). Speaking of *E. coli* mastitis isolates as a group of ExPEC strains that resemble UPEC in pathogenesis and invasive properties (Kaipainen et al., 2002; Dogan et al., 2006 and 2012), one would expect them to be comprised of group B2 strains.

To summarize the composition of our datasets: First, the *E. coli* isolates of bovine mastitis and bovine faeces were mainly classified to groups A and B1 (unpublished data). This is in accordance to collections of mastitis isolates reported elsewhere (Blum and Leitner, 2013; Ghanbarpour and Oswald, 2010; Dogan et al., 2006). Secondly, we included the so-called IMIB collection, which includes a broad variety of isolates from various different pathotypes and phylogenetic groups. Thirdly, the 111 isolates included into the *in silico* whole genome sequence screening (Zude et al. 2013, **Table 11**) mirror the variety of the *E. coli* phylogenetic groups. Thus, our data indicate that among the *E. coli* isolates from mastitis and bovine faeces, the phylogroup B2 is underrepresented in comparison to the selection of isolates included into the *in silico* genome sequence analysis (Zude et al. 2013).

From our data, it can be concluded that AT proteins are less prevalent among the bovine *E. coli* populations and more specifically, among mastitis isolates. This supports either the idea that ATs do not play a crucial role in mastitis.

V.5. Differential gene expression of mastitis *E. coli* 1303 and ECC-1470 in the presence of MAC-T epithelial cells *in vitro*

In order to investigate aspects of host-pathogen interaction of *E. coli* 1303 and ECC-1470, *in vitro* cell culture experiments were performed. MAC-T immortalized bovine mammary gland epithelial cells were co-cultivated with mastitis *E. coli* isolate 1303 and ECC-1470, respectively. As a reference, the *in vitro* transcriptome of *E. coli* 1303 and ECC-1470 grown in cell culture medium without MAC-T cells was determined using DNA microarrays. In **Figure 15**, a schematic overview of the performed investigation procedure is given:

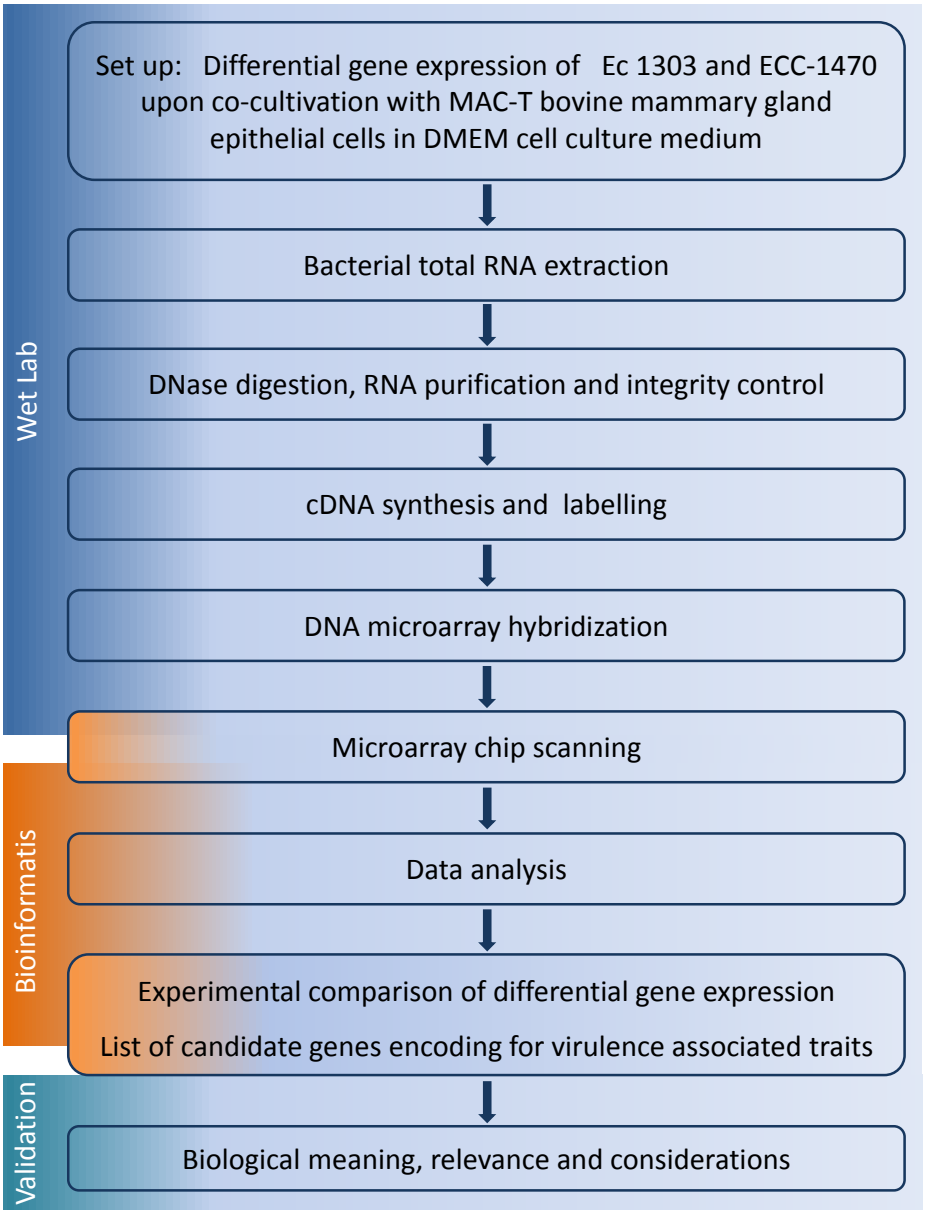

Figure 15: Basic work flow of the DNA microarray analysis performed to analyze the bacterial transcriptome. Samples of *E. coli* 1303 and ECC 1470 were acquired from cultivation at 37°C in DMEM in the presence or absence of MAC-T bovine mammary gland epithelial cells, respectively.

Study design appropriate to capture the transcriptomic response to present host cells

The cocultivation was performed according the culturing conditions described in section IV.2.3 . Briefly, approximately 1.5 x 10^7 MAC-T cells were present per cell culture flask during the cocultivation. The wells were infected with a MOI of 100. Therefore 2 x 10^9 bacteria of an overnight culture were co-cultivated with the MAC-T cells for one and three hours, respectively. Thus, bacteria had appropriate time to get into direct contact with the epithelial cells as we have also confirmed in section V.3.6. Total RNA samples were prepared from planktonic bacteria and bacteria in contact with MAC-T cells as described in sections IV.2.3 and IV.2.4. All DNA microarray experiments were performed in triplicates including the overnight culture. Comparative gene expression analysis was performed by DNA microarray hybridization with cy3- and cy5-labelled cDNA from total RNA extracts from *E. coli* 1303 and ECC-1470 grown in the presence of MAC-T epithelial cells relative to the absence of epithelial cells. A customized Operon Array was employed, which contains probe sets for all ORFs present in the genomes of *E. coli* strains MG1655, EDL933, O157:H7 Sakai, CFT073, 536 and UTI89. The results of the statistical analyzes were aligned to *E. coli* 1303 and ECC-1470 genome sequence data (Leimbach, unpublished data) and followed by the identification of differentially regulated genes at 1 h and 3 h after infection of the cell culture. Gene lists are provided in the appendix section in **Table 18**, **Table 25**.

V.5.1. Significant changes in the expression pattern

The mastitis *E. coli* strains 1303 and ECC-1470 exhibited differentially regulated genes when cultivated in presence of MAC-T bovine epithelial cells. Of approximately 5,155 CDS of *E. coli* 1303 and approximately 4,703 CDS of strain ECC-1470, we identified a total of 255 and 300 differentially regulated genes, respectively, during three hours cocultivation in the presence of MAC-T epithelial cells relative to the absence of epithelial cells. The individual number of up- and down-regulated genes at the different time points is shown in **Figure 16**. *E. coli* ECC-1470 exhibited a substantially higher total number of deregulated genes relative to *E. coli* 1303 at one hour time point.

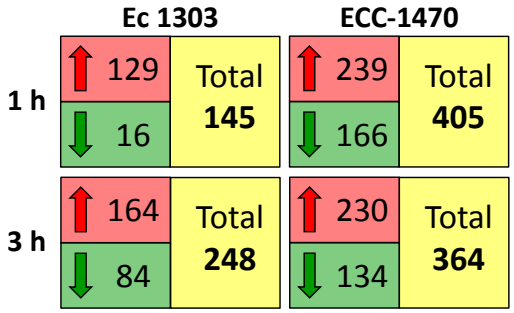

Figure 16: Deregulated genes in *E. coli* strain 1303 and ECC-1470 during cocultivation with MAC-T epithelial cells relative to the absence of epithelial cells. The diagram indicates the numbers of significantly up (red) and down (green) regulated genes ($P < 0.05$).

This is indicating a stronger transcriptional response of ECC-1470 to bovine mammary gland epithelial cells. Although the same parameters have been applied in all arrays an influence by poor microarray hybridizations cannot be excluded completely. The number of up-regulated genes exceeded twofold and that of the down-regulated genes exceeded tenfold the number of deregulated genes in *E. coli* 1303. At three hours after inoculation, the total number of genes deregulated by ECC-1470 decreased. At the same time the total number of genes deregulated by *E. coli* 1303 almost doubled upon ongoing cocultivation. Notably, the ratio between up- and down-regulated genes also differed considerably between both isolates (**Figure 16**).

Genes involved in metabolism represent one quarter of all deregulated genes

Differentially regulated genes were categorized according to a functional classification system (Hancock and Klemm, 2007) to summarize the main functional categories. As shown in **Figure 17**, the majority of the genes with significant changes during three hours of cocultivation clustered in the following functional categories: energy metabolism (22-28 %), proteins involved in intracellular transport and binding (11-16 %), cellular processes (9-10 %), DNA binding, replication, repair, restriction modification, transcription, RNA processing and degradation (8-10 %), and cell surface-associated proteins including such involved in transport and binding (7-9 %).

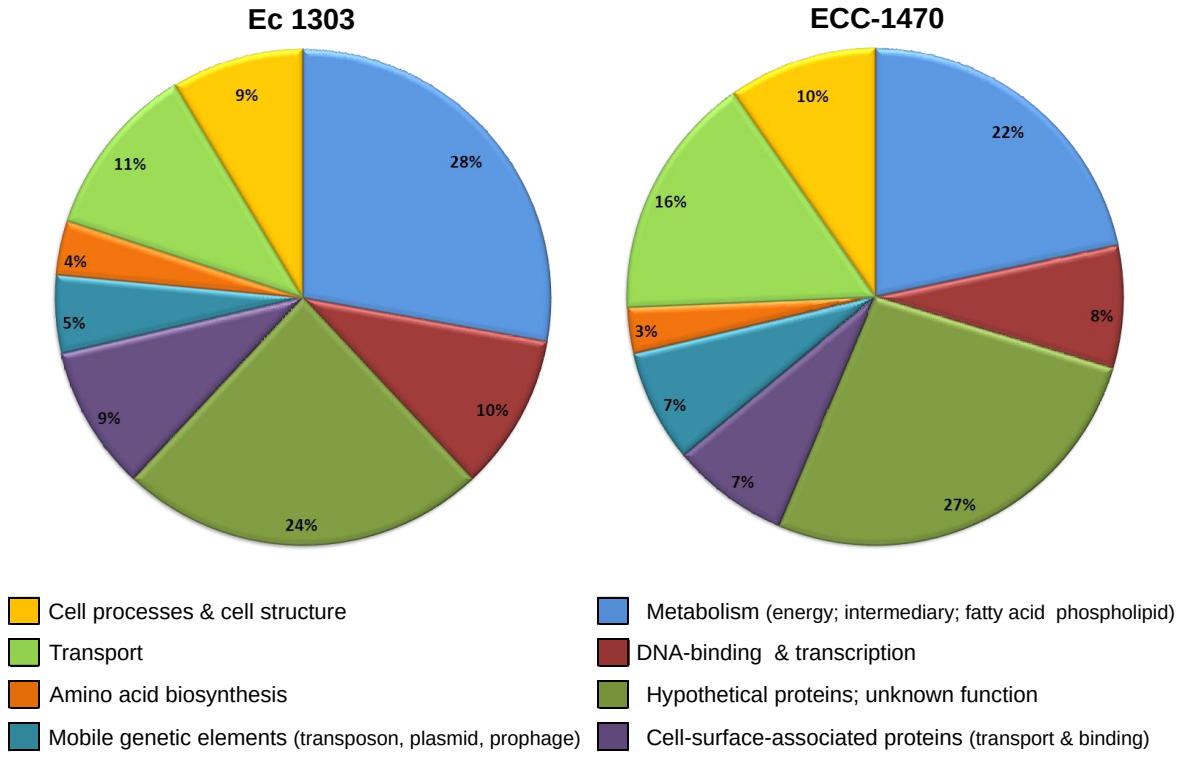

Figure 17: Functional classification of differentially up-regulated genes of mastitis *E. coli* 1303 and ECC-1470 during 3 h cocultivation in the presence of MAC-T epithelial cells relative to the absence of epithelial cells. Each slice of the pie chart represents a major functional group of genes. Numbers shown represent the percentage of the functional groups.

As in most microarrays, genes coding for hypothetical proteins or genes of unknown function accounted for a marked portion (24-27 %) of all differentially regulated genes identified. Genes that were functionally classified into the categories “amino acid synthesis” and “mobile genetic elements” represent the smallest portions of deregulated genes. At the first glance, *bona fide* virulence factors could not be identified among the significantly deregulated genes, though some genes are virulence-associated (see page 104). One could hypothesize, that strain ECC-1470 is not as dependent on metabolic adaptation as *E. coli* 1303, but rather deregulated transporters, probably to compensate for nutrient shortage due to the lack of other metabolic pathways. To confirm this hypothesis further investigation on nutrient utilization properties of both strains are required.

Commonly deregulated genes at 1 h and 3 h time in response to mammary gland epithelial cells

A hierarchical cluster analysis of the global transcription profile at one hour and three hours of the respective isolate indicated, that *E. coli* 1303 and ECC-1470 exhibited remarkable differences in their gene expression profiles as is shown in **Figure 18**. Genes that exhibited the same or a similar expression pattern were grouped together. The transcriptional response of the bacteria to the presence of bovine mammary gland epithelial cells resulted in commonly deregulated genes and, in case of *E. coli* 1303, revealed two subclusters whereas ECC-1470 showed five subclusters.

In both mastitis isolates, the majority of genes deregulated at either the 1-hour or 3-hour time point after inoculation differed in their transcriptional response by distinct expression levels or revealed no deregulation. Only a few genes of *E. coli* strain 1303 were commonly regulated at both time points whereas most genes were either regulated after one or three hours, but not at both time points. In contrast, *E. coli* ECC-1470 exhibited a higher number of commonly regulated genes as indicated by the bigger clusters. About 50 % of all deregulated genes of strain ECC-1470 were deregulated at one hour and also at three hours. A number of mainly down-regulated genes in *E. coli* ECC-1470 were either regulated at one or three hours but not at both time points. Altogether, this illustrates the very distinct transcriptional patterns of both strains. In *E. coli* 1303, the transcriptional patterns after one hour or three hours of cocultivation with bovine mammary gland epithelial cells were specific for both time points. The total number of deregulated genes almost doubled with ongoing cocultivation. In contrast, in strain ECC-1470 nearly half of the deregulated genes were commonly deregulated at both time points, indicating that the early and late transcriptional response was not that distinct in this strain. The mastitis isolates *E. coli* 1303 and ECC-1470 differ in their gene expression profiles in response to MAC-T cells. Whether this reflects the difference between a transient and a persistent lifestyle or whether this is a strain-specific phenomenon needs to be further investigated.

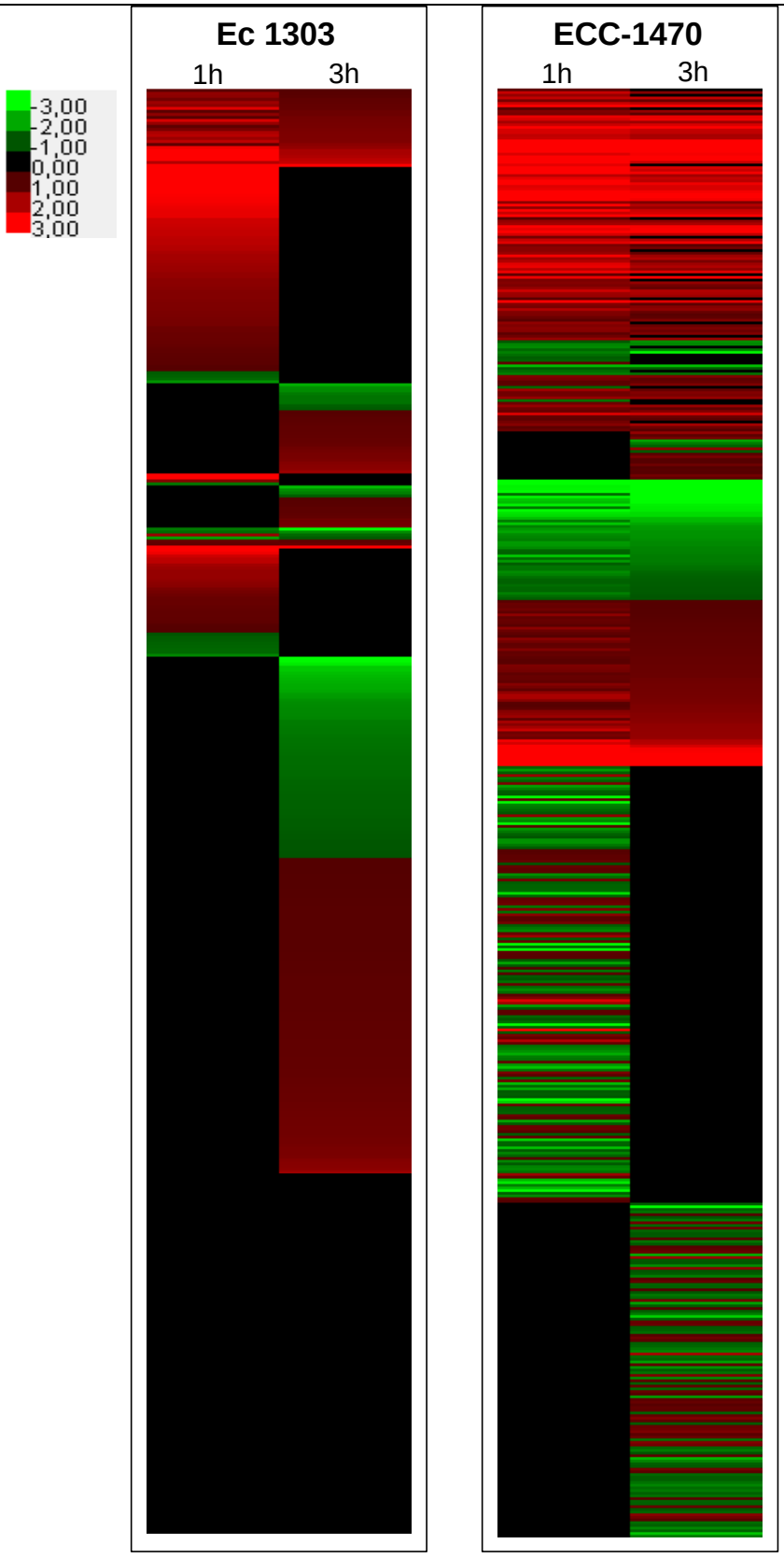

Figure 18: Transcriptional response of mastitis *E. coli* strains 1303 and ECC-1470 upon cocultivation with MAC-T cells. The hierarchical cluster plot shows the relative gene expression patterns of both strains upon cocultivation with MAC-T epithelial cells relative to the absence of MAC-T cells. Each bar represents one gene and its expression level corresponding to the color bar. The datasets for each strain and time point are mean values of the expression ratio from at least three independent microarray experiments. Genes without statistically significant changes in their expression profile are shown in black ($P < 0.05$).

The hierarchical cluster analysis revealed individually and commonly deregulated genes of each strain. Particular genes, which were commonly deregulated at both time points, might indicate the same bacterial reaction in response to the presence of bovine mammary gland epithelial cells. In contrast, individually deregulated genes might indicate strain-specific properties. Genes deregulated in both strains and possibly also deregulated within the same time period were examined and depicted in a Venn diagram shown in **Figure 19**. In addition to the visual output of the Venn algorithm a corresponding textual output is given in showing which genes in particular were deregulated at distinct conditions or were uniquely deregulated in a certain condition (data on compact disk: **Supplemental Table S1**). *E. coli* strain 1303 exhibited a comparably small number of individually deregulated genes after one hour of cocultivation relative to the other samples. Interestingly, the Venn diagram revealed 24 genes that were commonly deregulated after one and three hours of cocultivation in both strains *E. coli* 1303 and ECC-1470 and among them various regulatory determinants. Additionally, a considerable number of genes were commonly deregulated in both strains, but not necessarily at the same time as indicated by the overlapping areas of three samples (**Figure 19**). This suggests that the same processes are affected in both strains though up or down regulation in this Venn diagram has to be followed in the textual output in **Supplemental Table S1** on the compact disk. In **Figure 19** it is clearly shown that both strains individually regulated the transcription of a high number of genes at particular time points as indicated by the non-overlapping areas. These genes might represent strain-specific responses to the presence of bovine mammary gland epithelial cells.

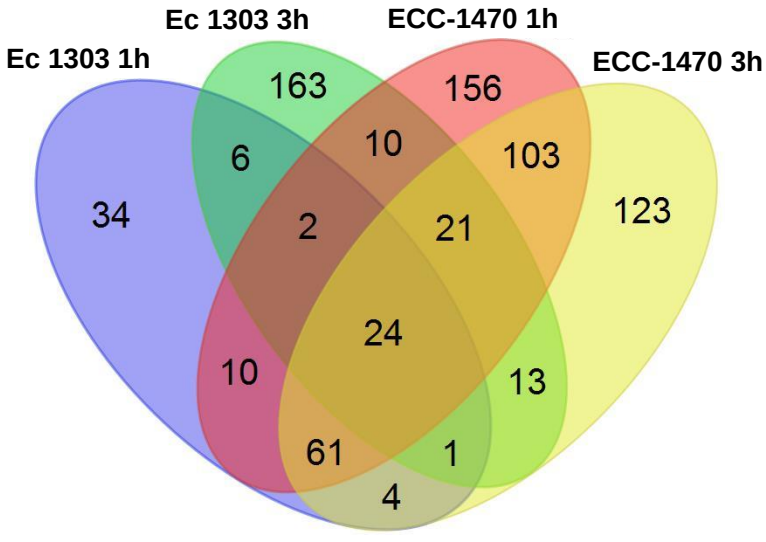

Figure 19: Commonly deregulated genes in bovine mastitis *E. coli* strains 1303 and ECC-1470 upon cocultivation with host cells *in vitro*. The Venn diagram shows the numbers of significantly deregulated genes in *E. coli* strains 1303 and ECC-1470 during cocultivation with MAC-T epithelial cells, relative to the absence of epithelial cells ($P < 0.05$). The numbers displayed in ellipses indicate the number of genes shared between the overlapping areas which represent the time of cocultivation and the strain, respectively.

It can be hypothesized that at least some of these genes might be involved in the different aetiological outcome caused by the transient *E. coli* strain 1303 relative to persistant strain ECC-1470.

V.5.2. Deregulated bacterial determinants in response to cocultivation with MAC-T cells

To better understand which cellular processes of mastitis *E. coli* 1303 and ECC-1470 were in particular reflected by the transcriptional response to the presence of MAC-T cells *in vitro*, we investigated representative deregulated transcriptional units and regulators selected from the Venn diagrams textual output (data on compact disk: **Supplemental Table S1**).The selected bacterial determinants and regulators that were deregulated during three hours cocultivation were depicted in **Figure 20**.

associated with…	1303	1303 & ECC1470	ECC1470
anaerobic conditions	*frdABCD* *abrB* *aer*	*hyaABCDEF* *hycABCDEFGHI* *hydN-hypF* *hyfABCDEFGHIJR-focB*	*napFDAGHBC-ccmABCDEFGH* *nikABCDER* *arcA*
energy metabolism	*deoCABD* *gudPXD* *paaABCDEFGHIJK* *galS* *ygbI*	*menFD-yfbB-menBCE* *malY* *rbsR*	*artPIQM* *astCADBE* *yiaLMNO-lyx-sgbHUE* *deoR*
extracytoplasmic stress	*ompR*	*tauABCD* *cpxP* *kdpD* *oxyR*	*gadBC* *rutR*
virulence	*motA & fliR*	*flhB* *baeS* *mdtG, mdtI, mdtL* *pixC* *Z4188* *mlrA*	*flhE* *hipAB* *yfcS* *Z4186* *csgD*

Figure 20: Selected determinants of mastitis *E. coli* strains 1303 and ECC-1470, which were up-regulated upon 3 hours of cocultivation with MAC-T cells. Black frames of the Venn diagram depict the overlap (number of shared features) of significantly deregulated determinants (blue), transcriptional regulators (red) and (green) down-regulated transcriptional regulator ($P < 0.05$).

Changes in energy metabolism indicating competition for nutrients

The majority of selected deregulated genes in **Figure 20** could be assigned to the category "Energy metabolism". In both strains we identified the ***menFD-yfbB-menBCE*** operon which is known to be specific to the menaquinone biosynthetic pathway (Dahm et al., 1998). Consequently up-regulated *men* expression suggests a preliminary production of menaquinones (vitamin K2 homologues), which can subsequently be used as the electron mediator between enzyme complexes involved in anaerobic respiration. Similarly, the expression of ***malY*** encoding a bifunctional protein with a regulatory and an enzymatic function (Reidl and Boos, 1991) was up-regulated in both strains. MalY is involved in the degradation of small carbon molecules and is a regulator of the maltose regulon. The transcription factor **RbsR** ("**R**i**b**os**e** **R**epressor") controls the transcription of the operon involved in ribose catabolism and is induced when *E. coli* is grown in the absence of glucose (Laikova et al., 2001; Mauzy and Hermodson, 1992). This indicates a competition between *E. coli* and host cells for glucose, which is the main carbon source in the DMEM cell culture medium. Thus, this finding supports on the one hand the idea that the bacteria responded to nutrient limitation during the cocultivation with MAC-T cells *in vitro*. On the other hand, *rbsR* is presumably vital *in vivo* as well, because it is also involved in the utilization of lactose as one of the main carbon sources in mammary secretions.

Apart from commonly up-regulated genes involved in energy metabolism, we evaluated those specifically expressed in either *E. coli* 1303 or ECC-1470. Interestingly, all the identified transcriptional units and regulators were involved in (i) carbon utilization under limited glucose availability (e.g. the *yia* and *gud* operons, the *deo* operon as well as the regulatory genes *galS*, *deoR* and *ygbI*) or (ii) utilization of amino acids as nitrogen source (e.g. arginine utilization by the AST pathway including the *ast* and *art* transcriptions units). With exception of the AST pathway, which was specific to strain ECC-1470, we did not observe any remarkable differences regarding the transcription of these genes between both mastitis strains. Accordingly, we demonstrate that three hours cocultivation with MAC-T cells resulted in glucose shortage and that *E. coli* consequently switches to other pathways involved in respiration and utilization of carbohydrates. This is further reflected by significantly up-regulated genes which are usually expressed under anaerobic conditions.

Deregulated genes reflecting anaerobic conditions during cocultivation

We identified up-regulated determinants and regulators that are functionally related to bacterial processes under anaerobic conditions (**Figure 20**). Of these, some were assigned to anaerobic conditions though they are also involved in the energy metabolism. This is exemplified by ***frd***, which encodes the fumarate reductase, an enzyme that catalyzes a reaction allowing fumarate to serve as a terminal electron acceptor when *E. coli* is growing under anaerobic conditions (Iverson et al., 1999). Thus, the *frdABCD* transcriptional unit was assigned to anaerobic conditions, but the encoded gene

products serve in energy metabolism. Nonetheless, our classification links the function to the conditions observed by the bacteria in the presence of MAC-T cells.

In both strains we identified the ***hyaABCDEF*** operon which codes for the *E. coli* hydrogenase 1 and which is induced under anaerobic conditions, but is repressed by nitrate (Richard et al., 1999). Interestingly, we identified the transcriptional regulator gene ***arcA*** to be up-regulated only in strain ECC-1470, but not in *E. coli* 1303. *ArcA* is known to antagonize repression of the hydrogenase-1 operon (*hyaABCDEF*) under anaerobic conditions (Nesbit et al., 2012). *ArcA* represent a transcriptional response regulator of two-component systems, which serve in bacterial signal transduction by sensing and responding to environmental stimuli and facilitate bacterial adaption to changing environmental conditions. In *E. coli*, the quinone-dependent ArcA/B two-component system mediates the response to changing respiratory conditions and functions as global transcriptional regulator under microaerobic and anaerobic conditions. When activated under anaerobic conditions, ArcA regulates the expression of numerous operons involved in respiratory and fermentative metabolism.

Also exclusively up-regulated in *E. coli* ECC-1470 was the ***napF*** operon encoding a ferredoxin-type protein. Another study reported a growth defect under anaerobic conditions on glycerol/nitrate medium when *napF* is missing (Brondijk et al., 2002). This suggests that NapF plays a role in energy conservation rather than a direct role in nitrate reduction. In this context it was also possible to successfully assign the other determinants ***hyd***, ***hyc***, and also ***hyf***, which were significantly up-regulated in both strains, to anaerobic nitrate respiration. In detail, the ***hydN-hypF*** operon is known to be maximally transcribed under anaerobic conditions in the presence of formate and is dependent on FhlA (Maier et al., 1996). In line with this, ***hycA*** which codes for the regulator of the transcriptional regulator FhlA was up-regulated as well. HycA controls the expression of several genes involved in the formate hydrogen lyase system. The specific regulatory mechanism by *HycA* is, however, unknown. It is known, that HycA expression is activated by formate and may interact directly with the FhlA protein and/or prevent the binding of FhlA to activator sequences (Suppmann and Sawers, 1994; Sauter et al., 1992; Leonhartsberger et al., 2000; Skibinski et al., 2002). We identified the ***hyf*** locus (*hyfABCDEFGHIJR-focB*) to be significantly up-regulated. The *hyf* locus encodes a hydrogenase-4 complex including a potential sigma (54)-dependent transcriptional activator HyfR (related to FhlA) and a putative formate transporter, FocB. *Hyf* expression is induced under fermentative conditions by formate at a low pH and FhlA-dependent. Its expression can be inhibited by HycA, the negative transcriptional regulator of the formate regulon (Skibinski et al., 2002). Supported by the notion that NAD-dependent formate dehydrogenases are important in prokaryotic anaerobic metabolism (Jormakka et al., 2003), our results clearly demonstrate that anaerobic metabolism was vital when the *E. coli* mastitis strains were co-cultivated with bovine host

cells. The comprehensive regulation of hydrogenase-associated genes was further supported by up-regulation of genes of the *nik* transcriptional unit encoding proteins essential for hydrogenase activity by nickel transport, which is required by *E. coli* when growing under anaerobic conditions (Rowe et al., 2005).

When it comes to regulators exclusively up-regulated in *E. coli* strain 1303, there is still little known about the regulatory gene ***abrB*** except that it might elevate the expression of the AidB CoA dehydrogenase when grown under anaerobic conditions. Because abrB mutations were too unstable for analyses *abrB* has not been further studied yet (Volkert et al., 1994). We also identified the well-examined regulatory gene ***aer*** to be up-regulated. Aer represents one of two sensory flavoproteins, which mediate the aerotactic response in *E. coli*. Specifically, the Aer protein senses the oxygen and energy state of the cell and mediates tactic responses towards rapidly oxidizable substrates such as ribose, galactose, maltose, malate, proline, alanine, glucose, mannitol, mannose, sorbitol, and fructose (Bibikov et al., 1997; Horne et al., 2009). Expression of *aer* by *E. coli* 1303 can be considered useful during mastitis pathogenesis due to the Aer-dependent activation of a number of genes involved in anaerobic respiration and the Entner-Doudoroff pathway (Prüss et al., 2003). Thus, increased Aer expression might enable quick utilization of available nutrients in mammary secretions.

Deregulated genes indicating extracytoplasmic stress

Another well-represented functional category of deregulated genes in *E. coli* strains 1303 and ECC-1470 represented ”extracytoplasmic stress” (**Figure 20**). This might result from either shortage of essential elements (e.g. sulfur starvation induces expression of the ***tauABCD*** operon) or inappropriate osmotic conditions, which induce expression of osmoregulatory determinants such as ***ompR*** and ***kdpD*** (van der Ploeg et al., 1996; Jo et al., 1986; Wood, 1999). The sensor kinase KdpD is part of the two-component system KdpD/E responsible for signal transduction in osmoregulation caused by changes in the intra- and/or extracellular K^+ concentration (Wood, 1999).

In particular, but not exclusively, vis-à-vis extracytoplasmic stress is indicated by up-regulated expression of ***cpxP*** encoding the regulator of the Cpx response and putative chaperone which is involved in resistance to extracytoplasmic stress. Specifically, *cpxP* transcription is induced by alkaline pH (Danese and Silhavy, 1998). This is on the one hand contradicting, because one would expect rather acidic than alkaline pH in cell culture resulting in expression of factors to maintain pH homeostasis and provide acidic resistance (e.g. ***gadBC*** and ***rutR***). This has been shown at least in case of *E. coli* ECC-1470. On the other hand, CpxP overproduction turns off the Cpx response by feedback inhibition (Raivio et al., 1999) supporting the notion that protection to alkaline conditions is not necessary. Interestingly, the Cpx system is also involved in adhesion and virulence of pathogenic *E. coli,* where *cpxP* expression is induced upon biofilm formation relative to planktonic growth in both exponential and stationary phase. In contrast isogenic *cpxP* mutants were impaired in biofilm

formation (Hung et al., 2001; Otto and Silhavy, 2002; Beloin et al., 2004). Moreover, the Cpx system may affect the bacterial ability to survive during or immediately after internalization. Alternatively, the invasion efficacy can be altered by transcriptional influence on how ExPEC modulate their surface characteristics (Debnath et al., 2013). More specifically, the Cpx system revealed the ability of UPEC to modulate P-fimbriae expression (Hernday et al., 2004; Hung et al., 2001; Jones et al., 1997). Therefore, it seems obvious, that deregulation of *cpxP* coding for a regulator of the Cpx response is involved in host-pathogen interaction.

The *E. coli* strains 13030 and ECC-1470 exhibited a strongly up-regulated expression of ***oxyR*** at one and three hours after inoculation. OxyR is a major transcriptional regulator for the expression of antioxidant genes in response to oxidative stress, in particular at elevated levels of hydrogen peroxide (Storz et al., 1990). The OxyR regulon comprises genes involved in peroxide metabolism, redox balance and protection against reactive oxygen species (ROS). Additionally, OxyR activates the synthesis of the small noncoding RNA *oxyS*, which regulates about 40 additional gene products (Storz et al., 1990; Zheng et al., 2001; Mongkolsuk and Helmann, 2002; Altuvia et al., 1997). The *oxyR* expression is induced during exponential growth by the cAMP-activated Crp protein, which we identified to be up-regulated in *E. coli* ECC-1470 during the three hours cocultivation. In contrast, *oxyR* expression is negatively regulated by RpoS when cells enter stationary phase (González-Flecha and Demple, 1997). Notably, *E. coli* 1303, but not ECC-1470 showed increased transcriptional levels of ***rpoS*** at three hours cocultivation. Recently, a novel function of OxyR unrelated to oxidative stress has been reported: OxyR might operate as regulator of a nitrosative stress regulon under anaerobic conditions exhibiting about 60 genes or operons dependent on OxyR under anaerobic conditions (Seth et al., 2012). We detected various other genes up-regulated during *in vitro* cocultivation with MAC-T cells, supporting the notion of anaerobic conditions meanwhile. Anyhow, whether OxyR is required for protection to either oxidative or nitrosative stress in our experimental setup has not been evaluated yet.

In summary, at least during *in vitro* cocultivation with MAC-T bovine mammary gland epithelial cells, the significantly up-regulated genes in both mastitis strains indicate extracytoplasmic stress. We emphasize, that this stress is probably caused by metabolic competition for nutrients and the secretion of products of metabolism into the cell culture medium by both, bacteria and host cells.

Expression of virulence-associated factors in response to the presence of MAC-T cells

In addition to up-regulated gene expression reflecting a more indirect response to MAC-T cells by adaptation to the changing environmental conditions during cocultivation, we have focused on the identification of up-regulated genes coding for virulence-associated factors. We identified two virulence-associated regulatory genes (***baeS*** and ***mlrA***) and different genes encoding virulence-associated factors (**Figure 20**).

Multidrug tolerance

The expression of the regulator-encoding gene ***baeS*** was up-regulated in both, *E. coli* 1303 and ECC-1470, at one hour as well as at three hours of co-incubation. The proteins BaeS and BaeR represent the sensor kinase and response regulator of the *E. coli* BaeS/R two-component system (Nagasawa et al., 1993). Both genes belong to an operon comprising the multidrug resistance gene cluster *mdtABCD*. BaeR binds to and thus stimulates the activity of the *mdtA* promoter (Baranova and Nikaido, 2002). Although we were not able to detect up-regulation of genes of the *mdtABCD* gene cluster, we identified several other genes encoding components of multidrug efflux systems to be up-regulated in both strains (e.g. ***mdtG***, ***mdtI***, ***mdtL***, (*mdt*= multi drug transporter)). It has been reported , that the overexpression of *mdtG* (also called *yceE*) and *mdtL* (also designated *yidY*), respectively, in a drug-sensitive background resulted in an up to four-fold increase in resistance to various antimicrobial agents (*mdtL*: deoxycholate and fosfomycin; *mdtL*: chloramphenicol, ethidium bromide and TPP) (Nishino and Yamaguchi, 2001). In contrast, *mdtI* is a yet uncharacterized components multidrug efflux transport system.

Fimbrial adhesins and flagella

In both mastitic strains ***mlrA*** was up-regulated, encoding the transcriptional regulator MlrA. The **M**erR-**l**ike **r**egulator **A** has been reported to facilitate curli production in an APEC strain and also in *Salmonella* (Brown et al., 2001). In APEC, the *mlrA* gene is regulated by RpoS, which we determined to be slightly up-regulated at three hours of cocultivation in *E. coli* strain 1303, probably to repress *oxyR* expression while bacterial cells enter the stationary phase. This might also affect the up-regulation of *mlrA*, which has been detected after three hours of coincubation in both strains, but exclusively at one hour cocultivation in *E. coli* ECC-1470. Consequently, upcoming enhanced expression of RpoS might result in *mlrA* down-regulation at three hours of cocultivation in *E. coli* strain 1303 and ongoing.

In contrast to the up-regulation of *mlrA* expression, we identified repression of genes involved in regulation, production and assembly of curli (***csgD***, ***csgC*** and ***csgF***) specifically in strain ECC-1470. Curli are encoded by the ***csg*** operon and it was previously suggested that these adhesins may be involved in the pathogenesis of bovine mastitis (Karczmarczyk et al., 2008). Moreover, curli are known to promote bacterial adherence to multiple cell lines. Curli fibers themselves interact with many host proteins and are potent inducers of the host inflammatory response (Barnhart and Chapman, 2006). Their role in mastitis pathogenesis remains unclear to date. The down-regulation of *csg* gene transcription including that of the transcriptional regulator CsgD in *E. coli* ECC-1470 after three hours coincubation with MAC-T cells is unexpected and differs from the situation in *E. coli* 1303. One would hypothesize that curli would be advantageous upon close contact to host cells. On the one hand, the down-regulation of curli gene expression in strain ECC-1470 in the presence of

cells indicates that there is no more need for curli. It may be that the persistent mastitis isolate ECC-1470 adopts upon prolonged coincubation with MAC-T cells to an inconspicuous lifestyle by progressive reduction of surface structures that became irrelevant in this advanced stage of cocultivation. On the other hand, it can be speculated, that energy became so limited that curli production is repressed for energy saving issues.

Besides the down-regulation of curli genes, we identified the up-regulated genes **ECP_2970** (in both strains) and **Z3599** (only in strain ECC-1470) encoding for components required for the biogenesis of fimbriae via the 'chaperone-usher' pathway. Z3599 encodes for **YfcS**, a fimbrial chaperone first identified in *E. coli* O157:H7 strain EDL933, while ECP_2970 encodes for fimbrial usher protein **PixC** first identified in *E. coli* 536. Whether common up-regulation of these fimbrial genes results in a functional fimbria of mastitic *E. coli* needs to be verified.

Another membrane-associated component, which might be involved in host-pathogen-interaction of mastitis *E. coli,* is the flagellum. We found significantly up-regulated gene expression levels (***flhB***, ***flhE***, ***fliR*** and ***motA***) in both strains, although not all of them were up-regulated at the same time (**Figure 20**). The *fliR* transcript was only identified in the early (1 h) transcriptional response of *E. coli* 1303 and encodes the flagellar biosynthesis protein FliR. FliR is one of the six integral membrane components of the flagellar export apparatus as is FlhB (Minamino and Namba, 2004; Fan et al., 1997; Kutsukake et al., 1994), which we identified to be up-regulated in both mastitis strains. Additionally, we identified *motA* that along with *motB* encodes the stator element of the flagellar motor complex (Ridgway et al., 1977). Moreover, we found *flhE* transcript levels to be up-regulated in *E. coli* ECC-1470. *flhE,* whose gene product may also be involved in the regulation of proton flow through the flagellar basal body, forms an operon with *flhB* and *flhA* which encode components of the flagellar type 3 export apparatus (Lee and Harshey, 2012; Liu and Ochman, 2007). It is, therefore, remarkable that although a statistically significant up-regulation of *flhE* and *flhA* transcription in *E. coli* 1303 could not be detected, this was the case for *fhlB* in both strains. This problem will be discussed in the next section, but in principal, we can state that we found evidence for the up-regulation of flagella in response to MAC-T bovine mammary gland epithelial cells. Thus, it can be presumed that flagella are involved in adhesion/invasion to mammary epithelial cells. Supporting this hypothesis, earlier studies reported that *E. coli* flagella were required for colonization of the gastrointestinal tract, including adhesion and subsequent invasion into intestinal epithelial cells of domestic animals (Xu et al., 2013; Duan et al., 2012 and 2013; Girón et al., 2002; Murinda et al., 2004). Besides the flagella-associated T3SS, we found up-regulated transcript levels of genes of another T3SS in *E. coli* strains 1303 and ECC-1470 during the early phase (1 h) of cocultivation. The genes Z4186 and Z4188 encode integral membrane protein-components of the ETT-2 type III secretion apparatus also present in *E. coli* O157:H7 strain EDL933.

Factors, which could be involved in the persistence of mastitis isolate ECC-1470

Interestingly, we identified the ***hipA*** *gene* to be up-regulated in *E. coli* ECC-1470. The *hipA* gene encodes the '**hi**gh **p**ersistence factor **A'**, a serine/threonine kinase mediating persistence and multidrug tolerance in *E. coli*. Except for a small fraction of dormant persister cells, *E. coli* would be killed by prolonged inhibition of peptidoglycan synthesis. But persisters are neither more resistant to inhibition of peptidoglycan synthesis, nor more likely to persist than normal bacterial cells. The molecular mechanism behind HipA-mediated persistence is to respond to the inhibition of the peptidoglycan synthesis by antibiotics (e.g. ampicillin) or by metabolic block. Thus, HipA induces consequent switching into a persister state of bacterial cell, characterized by growth arrest and strongly inhibited translation, transcription and replication (Moyed and Bertrand, 1983; Schumacher et al., 2012; Korch and Hill, 2006). Moreover, HipA, together with its antagonist HipB, are encoded by two genes organized as a transcriptionally autoregulated operon (*hipAB*), which constitutes a type II toxin-antitoxin locus. Most type II toxin-antitoxin loci are supposed to play a role in persistence and many of their mRNAs were significantly increased in persister cells (Germain et al., 2013; Keren et al., 2004; Shah et al., 2006). When the 11 type II toxin-antitoxin loci of *E. coli* were progressively deleted, a gradual reduction in persistence capability was detected, indicating their importance in *E. coli* persistence (Maisonneuve et al., 2011). We conclude that by monitoring the significantly up-regulated expression of *hipA* in response to MAC-T bovine mammary gland epithelial cells, we identified a bacterial factor, which, at least in part, contributes to the persistence capability of *E. coli* ECC-1470.

Factors, which could be involved in immune evasion of mastitis isolates

Significant up-regulation of ***wcaB*** and ***ypdI*** transcript levels was observed for both mastitis strains. The protein WcaB is believed to be an acetyl transferase involved in colanic acid synthesis based on sequence similarity and its presence in a colanic acid exopolysaccharide biosynthesis operon (Stevenson et al., 1996). Moreover, its expression was shown to be up-regulated in sessile bacteria relative to planktonic bacteria (Prigent-combaret et al., 1999). The same study reported that in addition to *wcaB,* also the *ompC* porin and the nickel high-affinity transport system-encoding gene *nikA* were stronger expressed in sessile bacteria. The expression of these genes was also up-regulated in the mastitis strains 1303 and ECC-1470 after 3 hour cocultivation with MAC-T cells. Like WcaB, the membrane lipoprotein YpdI has been shown to play a role in colanic acid synthesis (Stevenson et al., 1996), and its gene expression was also up-regulated in both strains. Together with the observed extensive induction of gene expression associated with microaerophilic and anaerobic conditions, we suggest that many of the initially planktonic bacteria turned into a sessile state and within a biofilm or microcolonies or in close contact with eukaryotic cells. Increased expression of genes involved in amino acid biosynthesis possibly releases nitrogen limitation or stress response

In both strains the expression of the ***hisLGDCBHAFI*** and ***ilvLXG*_1G_2MEDA** operons was up-regulated. The encoded gene products are involved in the biosynthesis of histidine, isoleucine and valine. It can be hypothesized that histidine, isoleucine and valine, which are glucogenic amino acids, might be converted into glucose through gluconeogenesis in order to compensate for the dwindling glucose in the cell culture medium due to the eukaryotic cells present.

We have not specifically evaluated the deregulated operons because they did not reveal any remarkably cellular processes which could be associated to the presence of MAC-T bovine mammary gland epithelial cells. Other up-regulated genes encode for proteins of arginine ABC transporter (**Ast**) and the arginine succinyltransferase pathway (**Art**) which is induced by nitrogen limitation and enables faster growth with arginine and aspartate (Schneider et al., 1998) or might be associated to stress response. A more detailed discussion on arginine metabolic pathways to stress response is discussed on page 150.

Valid analyses: misinterpretations and error sources in DNA-microarray analysis

Interpretation of "missing" genes in operons

We identified various two-component systems and different deregulated genes organized in operon structures. Unfortunately, some genes that were expected to be deregulated have not been determined although they belong to the same transcriptional units. Prokaryotic genomes are commonly organized in operons, meaning that in these operons several genes are co-transcribed resulting in "polycistronic mRNAs". It is known that a particular gene, which is transcribed as a part of a polycistronic transcript under one condition might also be individually transcribed as a single gene in another condition (Koide et al., 2009). Accordingly, it can be difficult to identify all members of operon structures under a given condition, but other explanations have to be considered as well. Alternative explanations for at least some of the non-detected genes are provided below, followed by a discussion of these data.

One possible explanation might be that some gene probes were simply missing on the microarray. Individual genes are not represented by corresponding probes, e.g. the *fhl* operon is not completely covered by probes. Although the arrays have been designed to cover the complete genomes of six *E. coli* strains (6 genomes and four plasmids), the individual genome annotations used at the production date were not as complete as they may be today. Consequently, this is one reason leading to individual "missing" genes.

Another explanation might be that some of the longmer oligonucleotide probes hybridized less properly. This may result in underrepresented signal intensity. Consequently, this adds bias to further analyses. The corresponding signal ratios might be excluded in statistical analyses due to repeated experiments or did not pass the stringent threshold. We suppose this explanation supported by following example: The genes *baeS* and *baeR* are part of an operon comprising the multidrug

resistance cluster *mdtABCD* of which we did not identified any gene to be deregulated under the conditions tested. Of the BaeSR two-component system, we identified only one encoding gene, *baeS*. According to the literature, *baeS* codes for the membrane-associated sensor kinase, which usually autophosphorylates in response to an environmental signal and transfers a phosphoryl group to the response regulator BaeR, which then becomes activated (Nagasawa et al., 1993). At the first glance this looks like an incomplete detection of the expression of the genes of this two-component system, but it truly is a misinterpretation due to bad signal quality. As a result, these bad quality signals for *baeR* were excluded from further analysis. While the BaeSR two-component system would not be functional with *baeS* expression alone, our assumption is corroborated by the significant up-regulation of *yicO* in strain ECC-1470. Expression of *yicO* is known to be positively regulated by BaeR (Baranova and Nikaido, 2002). This means, in consequence, that albeit significant changes in *baeR* transcript levels could not be detected by the microarray hybridization, increased *yicO* transcription indicates the expression of a functional BaeR/S two-component system.Similarly, the curli regulator-encoding gene *csgD* and some of the genes included in CsgD-dependent transcriptional units were also detected to be deregulated (*csgD*, *csgC* and *csgF*) while others seemed not to be significantly deregulated according to the microarray data (*csgE*, *csgG*, *csgA* and *csgB*). In **Figure 21** selected transcriptional units regulated by CsgD are shown. It was a general problem to interpret inconsistent results from microarray hybridizations, when particular genes were identified to be deregulated, but other genes of the same transcriptional unit have not been detected. By assessing the genetic organization of the operons and the analysis of corresponding regulons, we could gain sufficient information to predict other significantly deregulated genes that further supported our match. In this case, we also identified *wrbA* and *yccJ* to be deregulated based on DNA-microarray data. In total, we were able to identify the determinants and regulators presented in **Figure 20** by not only single indications, but due to corroborating results of other commonly deregulated genes.

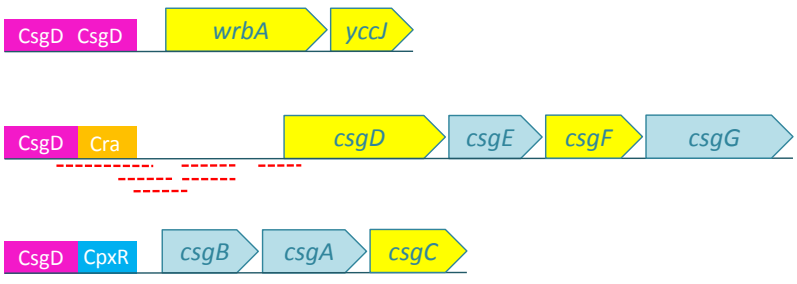

Figure 21: Schematic overview of selected transcriptional units regulated by CsgD. Genes highlighted (yellow) were deregulated during cocultivation upon MAC-T bovine mammary gland epithelial cells according to microarray hybridizations. Significantly deregulated transcript levels of genes colored in blue could not be detected. Elements are depicted as follows: Activator (magenta), inhibitor (orange), repressor (turquoise), small regulatory RNAs (red dashed line).

Good RNA quality and accurate microarray hybridizations, but bad signals: What happened?

Competitive DNA microarray hybridization involves numerous working steps accompanied by controls to ensure sample quality and attempts to normalize the cDNA amount of the competitive samples prior to the hybridization step. Our RNA isolation resulted in sufficient amounts of good quality RNA. We confirmed the RNA quality using the RNA integrity number (RIN). RIN values > 7.0 were considered to meet the requirements for gene expression analyses (Schroeder et al., 2006; Imbeaud et al., 2005; Wilkes et al., 2010). RNA concentration and sample purity of the preparations were determined using the NanoDrop® instrument and were considered sufficiently pure when the ratio A260/A280 was higher than 2.0.

So far, no obvious failures would have influenced competitive DNA microarray hybridizations. But what was not taken into account *a priori* was that total RNA samples were prepared from planktonic bacteria and from bacteria, which have been in contact with MAC-T cells as described in sections IV.2.3 and IV.2.4. Most of our initial array scans resulted in shifted ratio signals and bad signal-to-noise ratios. This specific problem was identified to result from eukaryotic total RNA contaminations present exclusively in the total RNA samples obtained from cocultivation with host cells, but from those cultivations in absence of host cells (**Figure 22**).

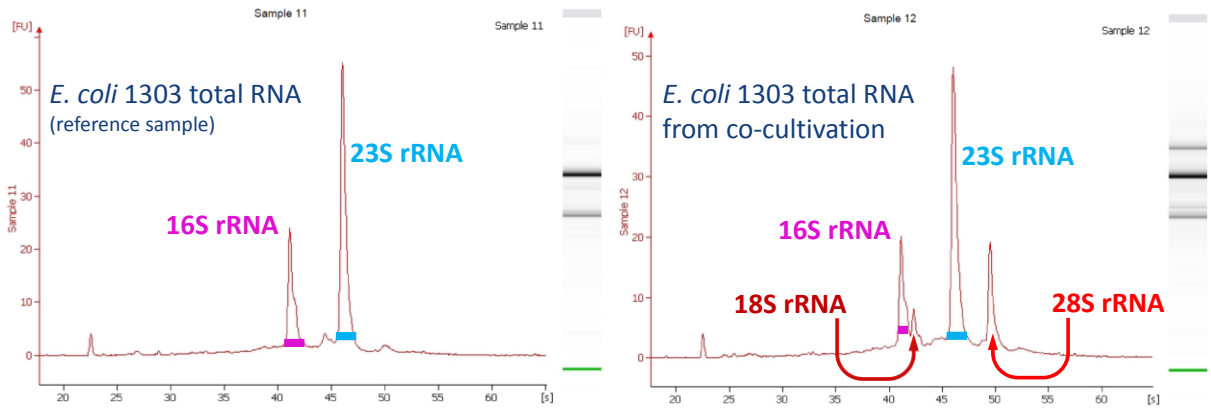

Figure 22: Quality control of bacterial total RNA isolated from *E. coli* 1303 cultures in the absence of host cells (A) relative to *E. coli* 1303 cultures from cocultivation with host cells (B). The RNA integrity curves/electropherograms and gel images are shown. Peaks indicate ribosomal RNA (rRNA) and residual eukaryotic rRNA is indicated by red arrows. The RNA integrity was determined by capillary electrophoresis using the Agilent 2100 Bioanalyzer instrument (RIN > 7.0).

Eukaryotic RNA cannot be completely avoided from RNA preparations with co-cultures of bacteria and host cells. As a consequence, the mixture of pro-and eukaryotic RNA negatively affects the competitive microarray hybridization. This results in slightly shifted signal ratios when attempting to

normalize labeled cDNA amounts of sample and reference sample. In our final microarray hybridizations we considered this effect. Nevertheless, we emphasize that this effect, which affected the bioinformatical analyses (**Figure 15**), had a lasting effect on ratio quality and subsequently resulted in semi-quantitative analyses. Consequently, we have not elaborated on quantification of deregulated gene expression, but made only qualitative statements regarding deregulation of genes. For the same reason we made no use of fold-change estimations. Fold change is often used, because of its simplicity (e.g. in rankings), but alone does not take into account variance and offers no associated level of confidence (Allison et al., 2006).

V.6. RNA-Seq: High throughput *E. coli* transcriptome sequencing

To comprehensively analyze the transcriptional response of the isolates *E. coli* 1303 and ECC-1470 in a setting mimicking the environment during initial pathogenesis, we cultivated the strains in native milk whey. Milk whey contains complement and defensins. As antimicrobial effects of raw milk and milk whey were observed, we used DMEM cell culture medium as a reference control mediating no antimicrobial effects, but allowing unrestricted bacterial growth. Moreover, the use of DMEM should allow comparison of the results of the RNA-Seq approach with those of the microarray-based approach to assess putative overlaps with the *in vitro* transcriptional response in response to cocultivation with bovine mammary gland epithelial cells (section V.5). **Figure 23** shows the general flow chart underlying the RNA-Seq experiment.

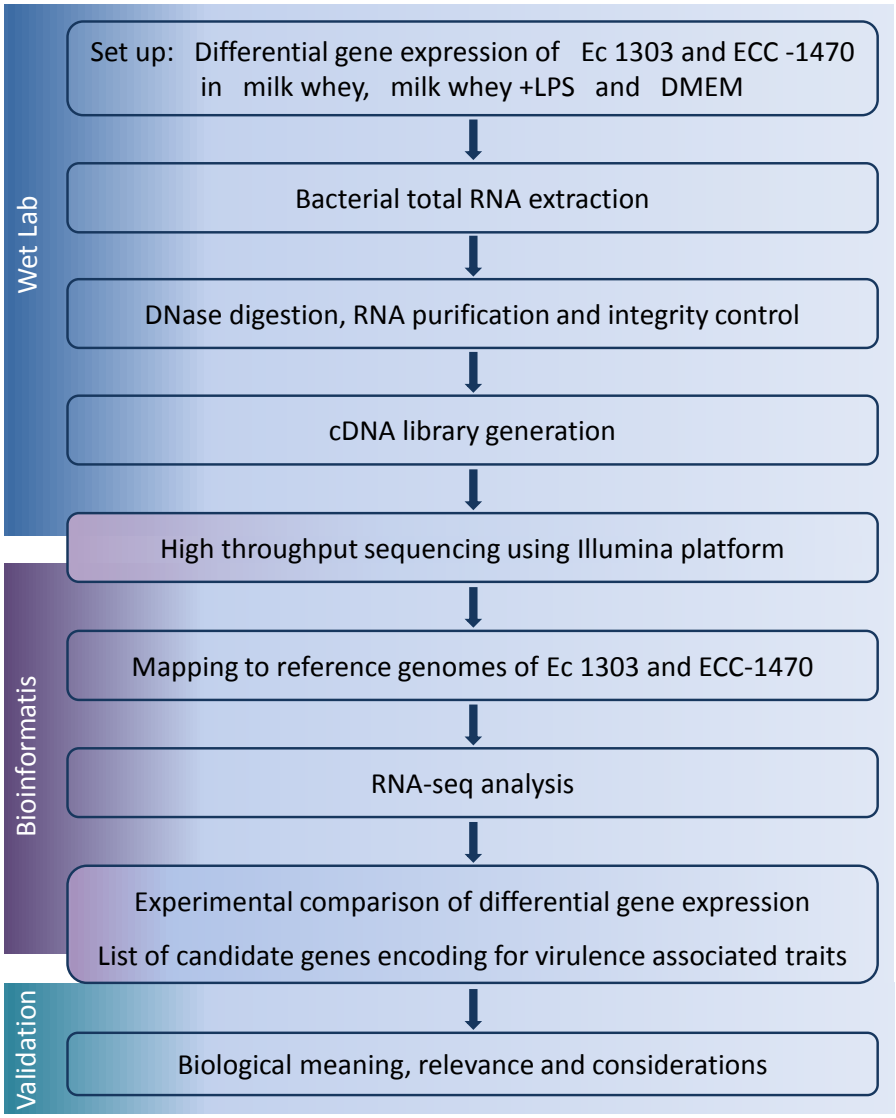

Figure 23: Basic flowchart of the RNA-Seq pipeline used to analyze the bacterial transcriptome in response to growth in raw milk whey relative to growth in DMEM. Samples of *E. coli* 1303 and ECC-1470 were taken upon cultivation at 37°C in DMEM, milk whey or milk whey from an udder challenged with LPS, respectively.

In this approach RNA-Seq was used to study differential gene expression of mastitis isolates *E. coli* 1303 and ECC-1470. The strains (1 x 10^9 CFU/ml of bacteria) were statically incubated for 1 h at 37 °C (body temperature) in three different media, respectively: i) milk whey from an udder-healthy cow, ii) milk whey from a cow´s udder infused with LPS, and iii) DMEM. Subsequently, RNA extraction, purification, mRNA enrichment and conversion to cDNA of the 6 samples, was performed before the cDNA libraries were sequenced using a HiScan sequencer (Illumina).

V.6.1. Quality of the sequence reads from *E. coli* transcriptome sequencing

The cDNA synthesis was carried out by vertis Biotechnologie AG and the sequencing of the cDNA pools was carried out at the core facility of the Leibnitz Institut für Arteriosklerosеforschung (LIfA), Münster following the Illumina protocol 1.7. The sequences of the sequencing reads obtained were demultiplexed and the barcode was trimmed. Per sample, a total of 15.6 to 31.9 million reads representing fragments of RNAs were obtained and further processed using the CLC Genomics Workbench 6.0.3 for identification of expressed genomic regions based on their read coverage.

Quality assessment and depletion of sequence reads covering rRNA genes

The resulting 101-bp sequence reads of the six cDNA samples were quality controlled with FastQC to assess the Sanger variant Phred scores (**Figure 24**). For example, a Phred score of 20 means that the base call is 99% accurate and vice versa a 1% error rate is equal to a Phred score of 20 (-10 x log 0.01). **Figure 24** shows that with increasing read length also the quality of the base call decreased. It is clearly visible that the RNA obtained from DMEM resulted in sequence reads with higher quality cDNA (Phred score < 20) until bp position 80, while those samples derived from milk whey and milk whey challenged with LPS merely reached bp position 70 with a Phred score > 20. This suggests that the specialized RNA extraction and purification protocol, required to obtain RNA from milk whey, is responsible for the difference in sequence quality. However, consistent sequence quality is important for comparative analysis. Consequently Phred scores below 20 (depicted by the red area) do not provide high discriminative power among base calls and were excluded. Therefore, the sequencing data were submitted to CLC Genomics Workbench 6.0.3 and quality trimmed with a cut-off Phred score of 20. Additionally, trimming of the sequence reads included removal of sequence beyond 90 bp of the sequences. The resulting sequences were filtered by length and sequences shorter than 15 nt were discarded. Assessing the Phred scores in this way guaranteed an ample read quality and additionally influenced the read mapping (section V.6.2).

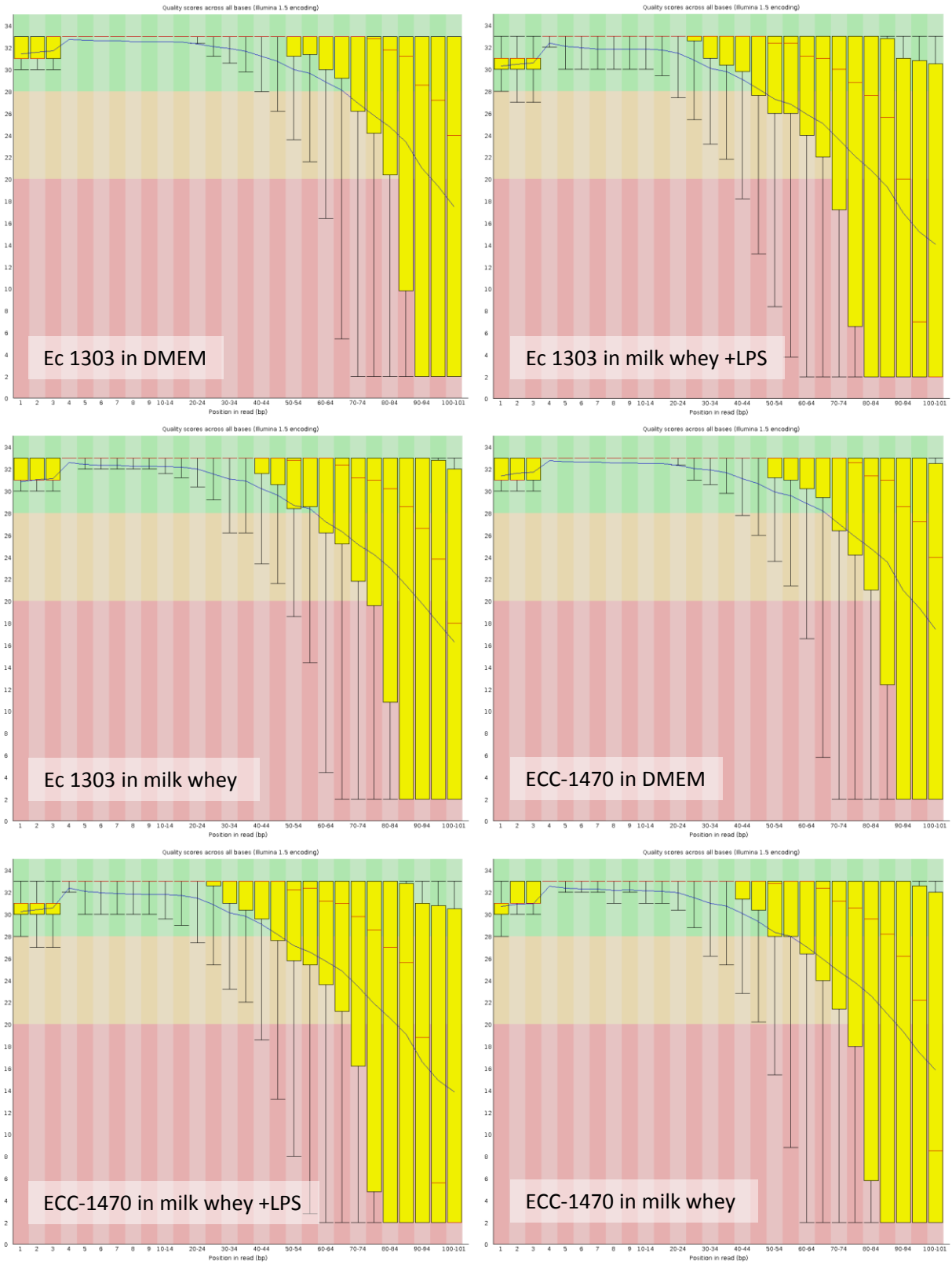

Figure 24: Read quality across all bases of the individual sequence reads. Sanger variant Phred scores mirror the sequence read quality of the six samples as assessed by FastQC software. The Phred scores *Q* assess the reliability of a base call and are defined as a property that is logarithmically related to the base calling error probabilities *P*. ($Q_{Sanger} = -10 \log_{10} P$).

V.6.2. Mapping of the sequence reads to the *E. coli* 1303 and ECC-1470 genome sequences

After read length adjustment and quality trimming the reads were mapped. The term mapping refers to an alignment of short sequence reads. Usually a reference sequence is used to which the reads can be mapped, but reads might also be assembled *de novo* what refers to the computational reconstruction of a longer sequence from smaller sequence reads. Generally mapping assemblies, even to rather phylogenetically distant reference genomes, provided more accurate gene expression levels and outperformed differential expression inference of *de novo* assemblies (Vijay et al., 2013).

The specific reference genome sequences of *E. coli* isolates 1303 and ECC-1470 (Leimbach unpublished data) were used to map the sequence reads of these strains, respectively. Initial mappings revealed that up to 63 % of the obtained total reads covered ORFs encoding for rRNA genes. As normalization between the different samples by the amount of reads or bases is included in downstream data analysis, we filtered the reads covering genes of 16S and 23S rRNA according to Kopylova et al., 2012. Following this procedure, between 7.7 and 14.6 million reads were obtained per sample for further transcript analysis.

These remaining high-quality sequence reads of each sample were again submitted to CLC Genomics Workbench 6.0.3 and mapped to their respective reference sequence. Reads that were not removed by the rRNA read filtering, but which mapped to one of the 22 rRNA genes (*rrf*ABCDEFGH*, rrl*ABCDEGH and *rrs*ABCDEGH) were removed manually. The selected read mapping parameters were modified to fit the combination of the used cDNA synthesis method, Illumina sequencing protocol and prokaryotic reference sequence obtained by 454 sequencing, because most protocols and preset mapping parameters are adjusted to handle data obtained from eukaryotic organisms with defined and fully annotated reference sequences. The method to obtain the reference genome sequences of *E. coli* 1303 and ECC-1470 and its annotation status at that time greatly influenced the outcome of the present analyses.

Table 12 includes the summarized mapping statistics and **Figure 25** shows the match specificity of our RNA-Seq data. Sequence reads matching zero (0.0) times failed the mapping parameters (section *.0.0 mapping*) although they can be mapped to the reference sequence. Between 5.0 and 6.8 % of the total reads per sample can be referred to as counted reads defining the reads that fit the parameters. They can be distinguished into specific and non-specific reads. The vast majority are "specific counted reads" which matched to only one position in the reference sequence. In contrast, the number of reads, which were equally matched to other positions in the reference sequence, is called "non-specific counted reads". This histogram shown on **Figure 25** serves as an example of the match specificity acquired for all samples. The match specificity shown in **Figure 25** graphically exemplifies the number of reads that match, by the chosen parameters, at one or more positions. It has been demonstrated that approximately 0.99 - 1.97 million reads could be correctly mapped.

Table 12: Summary of mapping statistics from RNA-Seq data

Sample		Total reads	Reads after rRNA read filtering	Counted reads	Specific counted reads	Non-specific counted reads
	DMEM	25,392,624	14,583,645 [57.4%]	1,376,757 [5.4%]	1,256,305 [91.3%]	120,452 [8.7%]
Ec 1303	milk whey	31,590,658	11,770,074 [37.3%]	1,567,442 [5.0%]	1,379,462 [88.0%]	187,980 [12.0%]
	milk whey +LPS	30,328,686	11,218,479 [37.0%]	1,969,927 [6.5%]	1,713,996 [87.0%]	255,931 [13.0%]
	DMEM	20,405,112	13,007,005 [63.7%]	1,181,715 [5.8%]	1,085,496 [91.9%]	96,219 [8.1%]
ECC-1470	milk whey	22,780,194	12,453,415 [54.7%]	1,559,539 [6.8%]	1,405,507 [90.1%]	154,032 [9.9%]
	milk whey +LPS	15,543,923	7,703,133 [49.6%]	998,786 [6.4%]	896,847 [89.8%]	101,939 [10.2%]
			% refers to total reads as 100%		% refers to counted reads as 100%	

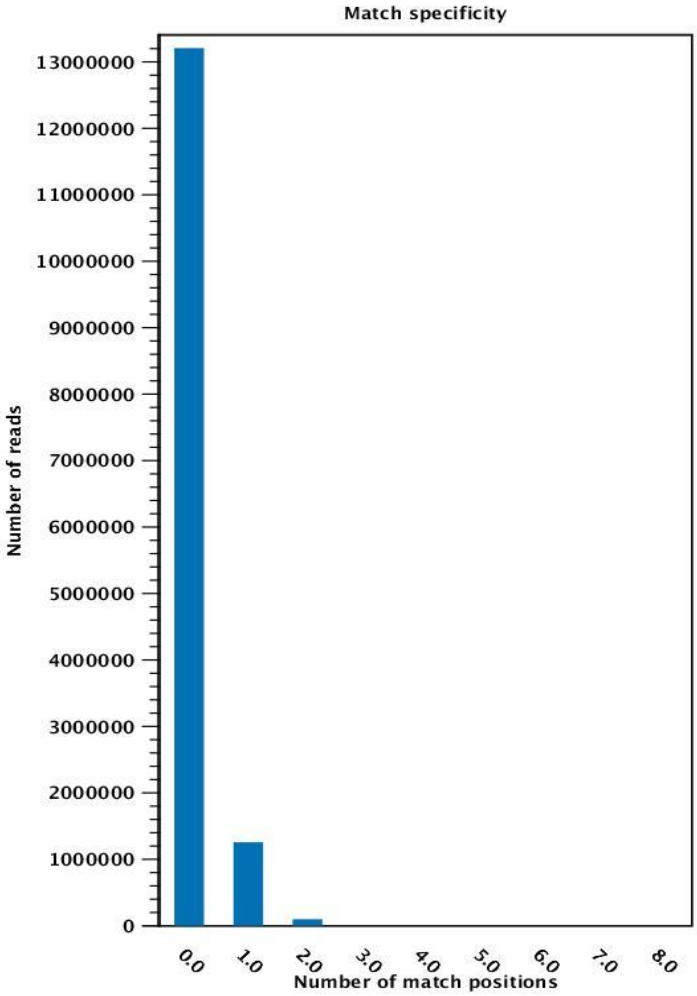

Figure 25: Match specificity of sequence reads from cDNA sample "*E. coli* 1303 cultivated in DMEM". Uncounted reads: The sequence reads matching zero (0.0) times. Specific reads: The sequence reads that match one position (1.0) in the reference sequence. Non-specific reads: Sequence reads that match equally to more than one position in the reference sequence (2.0 to 8.0 positions).

Sequencing depth of the cDNA samples

The optimal sequencing depth is varying based on the study´s objective. As we did not intend to characterize the full transcriptome including all weakly expressed genes, but to analyze the differential expression of genes (DEG), we questioned whether the achieved sequencing depth is deep enough.

In the present analysis, we achieved a 16-fold genome coverage of *E. coli* 1303 corresponding to a per sample average of 328 reads per gene (at 5,155 annotated genes). In case of *E. coli* ECC-1470 a per sample average of 249 reads per gene was achieved corresponding to a 19-fold genome coverage (at 4,703 annotated genes). Notably, counted reads have been mapped to annotated genes exclusively.

In order to allow for comparison with other studies, we provided the sequencing depth as sequence reads per gene and fold coverage of the coding genome sequences. In contrast to a coverage exceeding 10 million reads per sample, when analyzing comprehensive transcriptome profiles, it has been reported that 5 to 10 million reads per sample are sufficient for most applications of *E. coli* RNA-Seq (Haas et al., 2012). It has further been reported that 4 million non-rRNA reads were sufficient to identify unannotated genes of *V. cholerae* or that even 30 thousand non-rRNA reads allowed for identification of only twofold deregulated genes in *E. coli* EDL933 (Mandlik et al., 2011; Haas et al., 2012).

In our RNA-Seq experiments, we acquired a sequencing depth of 7.7 to 14.6 million non-rRNA reads per sample after filtering (**Table 12**). Out of these, 9.1 to 17.6 % turned out to be mappable reads according to the final mapping parameters. Thus, 0.99 to 1.97 million “counted reads” could be correctly mapped providing accuracy and inferential power for the present RNA-Seq-based identification of differential gene expression. The achieved sequencing depth is appropriate.

One may wonder why only 5.0 to 6.8 % of the total reads or 9.1 to 17.6 % of the non-rRNA reads were mappable. Why could so many reads not be aligned to the respective reference sequences?

In fact, a vast number of reads would be mappable and considered “counted reads” upon modification of the mapping parameters. Instead, we decided for more stringent parameters in order to reduce the proportion of erroneously mapped reads and to identify truly differentially regulated genes even if their expression was not markedly deregulated. As discussed earlier, the efficacy of extraction and purification of the total bacterial RNA and the quality of cDNA synthesis remain variables in transcriptome analyses. They affect the outcome of the RNA-Seq read data quality (**Figure 24**). This causes interference between the sequence identity of the read and the reference sequence. In addition, combining different sequencing technologies might considerably affect the study by adding bias to the bioinformatical processing during both mapping and read allocation.

Effect of non-specific read matches on gene expression levels

The extent to which a particular gene is transcribed into mRNA is indicated in expression levels. Analog expression data, such as those derived from competitive DNA microarray hybridizations, reveal the ration of transcript levels present under the two different conditions compared, whereas the expression level derived from RNA-Seq is measured by the number of sequence reads mapped to the reference genome of the individual cDNA sample.

A major issue in selecting the appropriate mapping parameters is whether to include non-specific reads in the RNA-Seq analysis or not. Non-specific reads match equally well to more than one position on the reference genome sequence. Therefore, taking non-specific reads into account might cause serious failure in computation of gene expression levels, but cannot simply be discarded, because the expression of the assigned genes will be underestimated or not even be reported (Roy et al., 2011). In contrast, including non-specific but possibly mismapped reads would result in an overestimation of gene expression levels as well. This raises the question as how to handle non-specific reads in the present study?

The sample´s read mappings to the respective reference genome were performed with both i) parameters set for including specifically matching reads only and ii) parameters set for including non-specific reads. The former mapping algorithm (i) discards ambiguously mapped reads and only keeps uniquely mapped reads. The latter mapping algorithm (ii) distributes non-specific reads in relative proportion according to the number of uniquely mapped reads of the assigned features, normalized by feature length. Basically this means that first the expression of each gene is estimated by reads that map uniquely and then this information is used to weight the distribution of the non-specific reads.

In order to visualize how this mapping algorithm qualitatively and quantitatively affected the computation of expression values, a scatter plot was created for each sample (**Figure 26**). The scatter plots show the reads match specificity by plotting the expression levels (number of total reads mapped per feature/gene) of a mapping with only specific reads against the same sample mapped with inclusion of non-specific reads. Since the mappings were run on the same data set with the only difference being the consideration of non-specific reads, one can focus on the very few features influenced by the read distribution. Most genes had close to identical expression values what was expected due to the utilization of the same data set. However, some genes in the mappings including non-specific reads exhibited much higher expression levels (as indicated by the red spots in **Figure 26**). But also the visible portion of outliers is rather small compared to the number of total genes for each of the reference genomes (Ec 1303 comprises 5,155 genes; ECC-1470 comprises 4,703 genes).

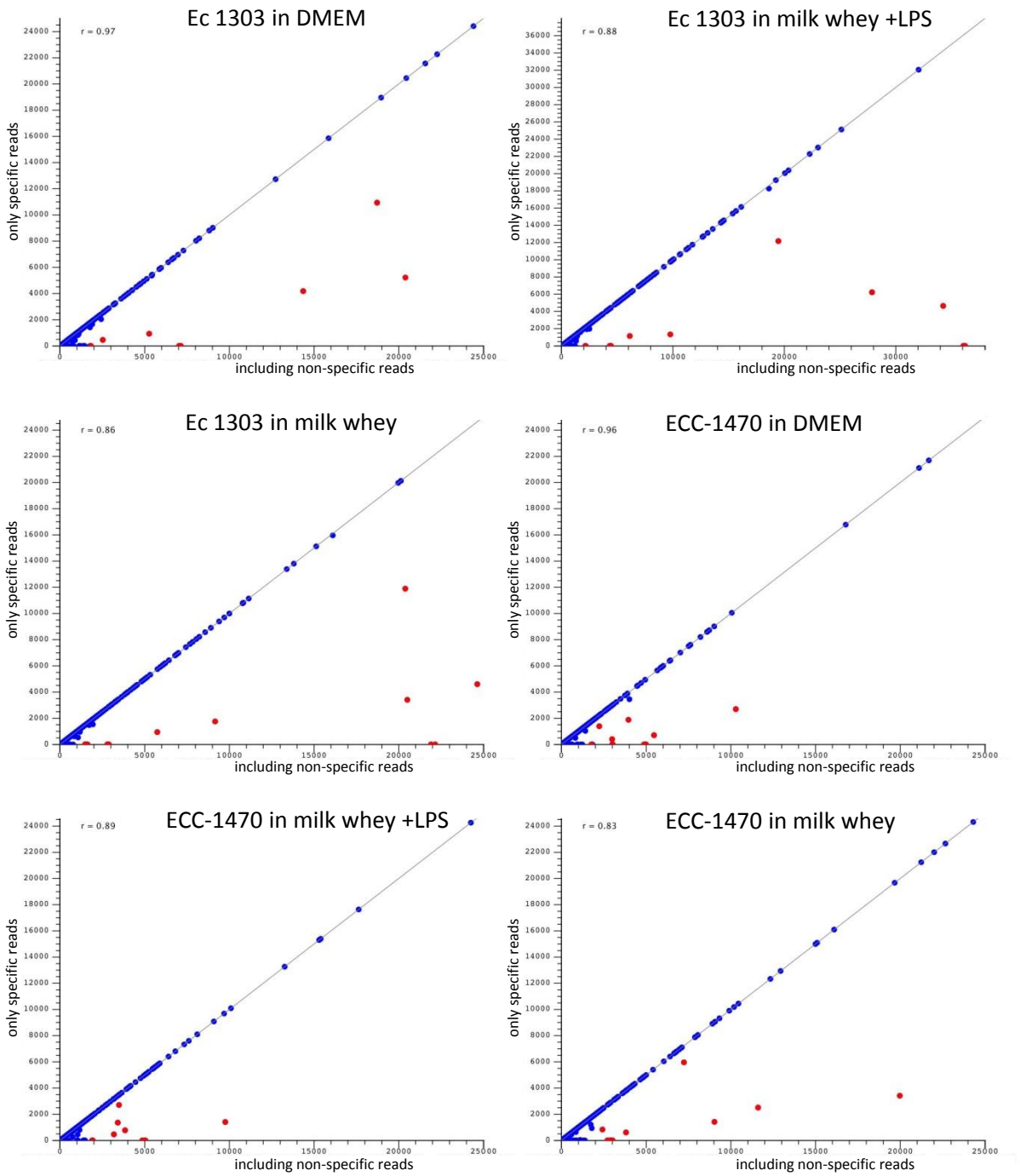

Figure 26: Effect of non-specific read matches on quantification of gene expression levels. Expression levels were depicted in scatter plots. Blue spots indicate similar read counts revealing similar estimated expression levels and red spots highlight specific outliers between mappings of only specific reads relative plotted against mapping including non-specific reads. The scatter plots were created from expression data (read counts) of annotated genes.

The expression levels of only a few genes (outliers) were vastly increased because of the total number of reads assigned including non-specific reads. These genes are suggested to be either paralogous genes, pseudogenes instead of highly expressed functional genes or genes containing many repeat regions, which are most likely uniquely covered by specific reads. In fact, some of these genes have a repeat-rich region (e.g. the *rhs* genes). Furthermore, some genes are partly covered by

higher numbers of non-specific reads due to overlapping regions (e.g. *flmC* and *flmA*), which means that all reads that map to the *flmC* gene, also map equally well to the overlapping part of the other gene (*flmA*).

In bacterial genomes, neighboring genes can often overlap. Therefore, distinguishing the start of one transcript from the end of another transcript adds complexity to transcriptome analysis (McClure et al., 2013). At least in part, this obstacle was overcome by the utilization of a strand-specific protocol for read generation allowing a valid distribution of reads to the overlapping genes through detection of overlapping transcripts coded in opposite orientations.

Another explanation arises from incorrectly sequenced homopolymer stretches, which are common artefacts of the 454 sequencing technology (Wolf, 2013), which has been used to generate the reference genome sequences of *E. coli* strains 1303 and ECC-1470 (Leimbach unpublished). In contrast, the sequence reads of the RNA-Seq samples were derived from Illumina sequencing technology. The reference 454-generated genome sequences were more prone to miss-alignment and therefore affected regional read coverage. In contrast, the sequence reads obtained from the Illumina platform were not that affected by incorrectly homopolymer sequences. This causes a bias during Illumina-read alignment and read allocation to a sequence of 454-origin. Consequently, the mapping parameters have to allow for some variability to enable a sufficient coverage at all. Generally, this issue resulted in a lower number of total reads mapped, but reads that covered intercepted genes due to bad reference sequence quality were then determined non-specific reads either. One option to overcome this problem would be to (yet manually) correct the alignment using the Illumina data. As this study focused on differential gene expression, this issue has not necessarily to be addressed and it has thus not been considered for further analysis in this study.

Including non-specific reads

In all samples, the most outlying gene was *flmC*, which was not in the range of the plot scale. The *flmC* gene product is known to modulate and start translation of host killing protein FlmA. Assigning a great number of non-specific reads to *flmC* would lead to a marked overestimation of expression and thus to an erroneous interpretation. To avoid misinterpretations, the genes whose expression levels exhibited a marked alteration upon the inclusion of non-specific read matches have been identified and listed according to their presence in each of the samples. Furthermore, the intersections from each sample-list containing genes of markedly altered expression levels have been determined.

Table 13 lists which genes were in each intersection or were unique to a certain sample. The enormously high *flmC* expression level would lead to the expectation that also *flmA* expression levels were high. This was true in all of the six samples tested. In addition, *srnB* transcription has been identified to be up-regulated. *snrB* is supposed to be a paralogous gene showing similarity to *flmC*, but also to the *mokC* gene. The latter gene was identified to be markedly expressed only in the

ECC-1470 milk whey sample. The genes (*srnB, flmC,* and *mokC*) share sufficient sequence similarity to allow mismapping of non-specific reads.

Furthermore, *tufA* and its duplicate *tufB* code for elongation factor Tu (EF-Tu) and also showed enormously high expression levels. EF-Tu is considered to be the most abundant protein in *E. coli* while being maximally expressed during stress response (Muela et al., 2008). The expression of the aforementioned *tufA* and *tufB* genes in all samples would add an interesting aspect to the present analysis of differential gene expression. They, however, have to be excluded from further analysis due to the following reasons: i) any expression measure of a paralogous gene will be biased because currently there is no mapping algorithm available that can correctly include sequence data from paralogues, and ii) misestimated expression levels can neither allow nor support a reliable identification of differential gene expression.

Table 13: Non-specific read matches which led to markedly altered gene expression levels under different growth conditions

Sample / Intersection	Total genes	Annotated CDS
Ec 1303 DMEM *Ec* 1303 milk whey *Ec* 1303 milk whey +LPS ECC-1470 DMEM ECC-1470 milk whey ECC-1470 milk whey +LPS	5	*srnB, tufB, flmA, tufA, flmC*
Ec 1303 DMEM *Ec* 1303 milk whey *Ec* 1303 milk whey +LPS	3	EPE_c45090, EPE_c23190, EPE_c27300
ECC-1470 DMEM ECC-1470 milk whey ECC-1470 milk whey +LPS	1	ECN_c20070
Ec 1303 milk whey *Ec* 1303 milk whey +LPS	6	EPE_c01610, EPE_c28560, EPE_c28550, EPE_c28540, EPE_c28530, EPE_c01630
ECC-1470 DMEM ECC-1470 milk whey	3	ECN_c13870, ECN_c07910, ECN_c07890
ECC-1470 milk whey ECC-1470 milk whey +LPS	3	ECN_c24260, ECN_c24250, ECN_c24270
Ec 1303 DMEM	2	*tnpA*, EPE_109p0260
Ec 1303 milk whey	6	EPE_c01640, EPE_c16190, EPE_c14090, EPE_c13670, EPE_c01650, EPE_c29260
Ec 1303 milk whey +LPS	2	EPE_c31140, EPE_c31150
ECC-1470 DMEM	6	EPE_c24260, EPE_c24250, ECN_c11680, ECN_c42470, ECN_c12070, EPE_c24270
ECC-1470 milk whey	2	*mokC*, ECN_c07900
ECC-1470 milk whey +LPS	4	ECN_c25580, ECN_07900, ECN_c25590, *ydcM*

Of all CDS that exhibited markedly altered expression levels upon inclusion of non-specific reads (**Table 13**), particular genes might be of interest for our analysis of genes contributing to mastitis. Specifically, genes that are present in an intersection of the Venn diagram comprising neither all

samples, nor all strain-specific samples (here growth conditions), or are unique to a certain sample. Their altered gene expression, even if biased, seemed to be exclusively altered in particular samples or under specific conditions mirrored by their allocation to individual intersections. **Table 13** summarizes a selection of genes, which for the above mentioned reasons should be further investigated.

There is, nevertheless, disagreement in the literature on whether to include non-specific reads in the RNA-Seq analysis or not. Aiming to discover mastitis-relevant candidate genes by analysis of differential gene expression rather than by performing a quantitative global expression analysis, the bias does not necessarily compromise our analysis. The consideration of including non-specific reads might avoid overlooking certain genes. Hence, we decided that the approximately 8-12 % non-specific reads per sample (**Table 12**) should be included into the downstream RNA-Seq analysis.

Read distribution, variability and similarity across samples

The different probe sets had to be examined according to their overall read distribution, variability and similarity. Therefore, the original expression values (total reads per gene) had to be transformed or normalized in order to allow comparability across samples.

Sample distributions of gene expression levels

When analyzing gene expression values of different genes within a sample, the coding sequence (CDS) of all the genes is usually not of the same length. A long transcript is, therefore, expected to be covered by more reads than a short transcript of equal expression level. Thus, instead of the original expression values (total reads per CDS) the 'reads per kilobase of gene' model (RPKM) was applied, which takes the gene length into account (Mortazavi et al., 2008). Additionally, the data were $\log_2$-transformed in order to remove the expression values´ dependence on the variance of the mean. The distributions of the $\log_2$(RPKM) expression values for the 5,155 genes of *E. coli* 1303 and 4,703 genes of *E. coli* ECC-1470 defined as annotated CDS are shown in **Figure 27**. All RPKM distributions are approximately normal. A uniform read coverage across the annotated CDS is not provided, what is in accordance to previously performed RNA-Seq analyses on both, eukaryotic and prokaryotic organisms (Kertesz et al., 2010; Wilhelm et al., 2008; Chaudhuri et al., 2011; Perkins et al., 2009).

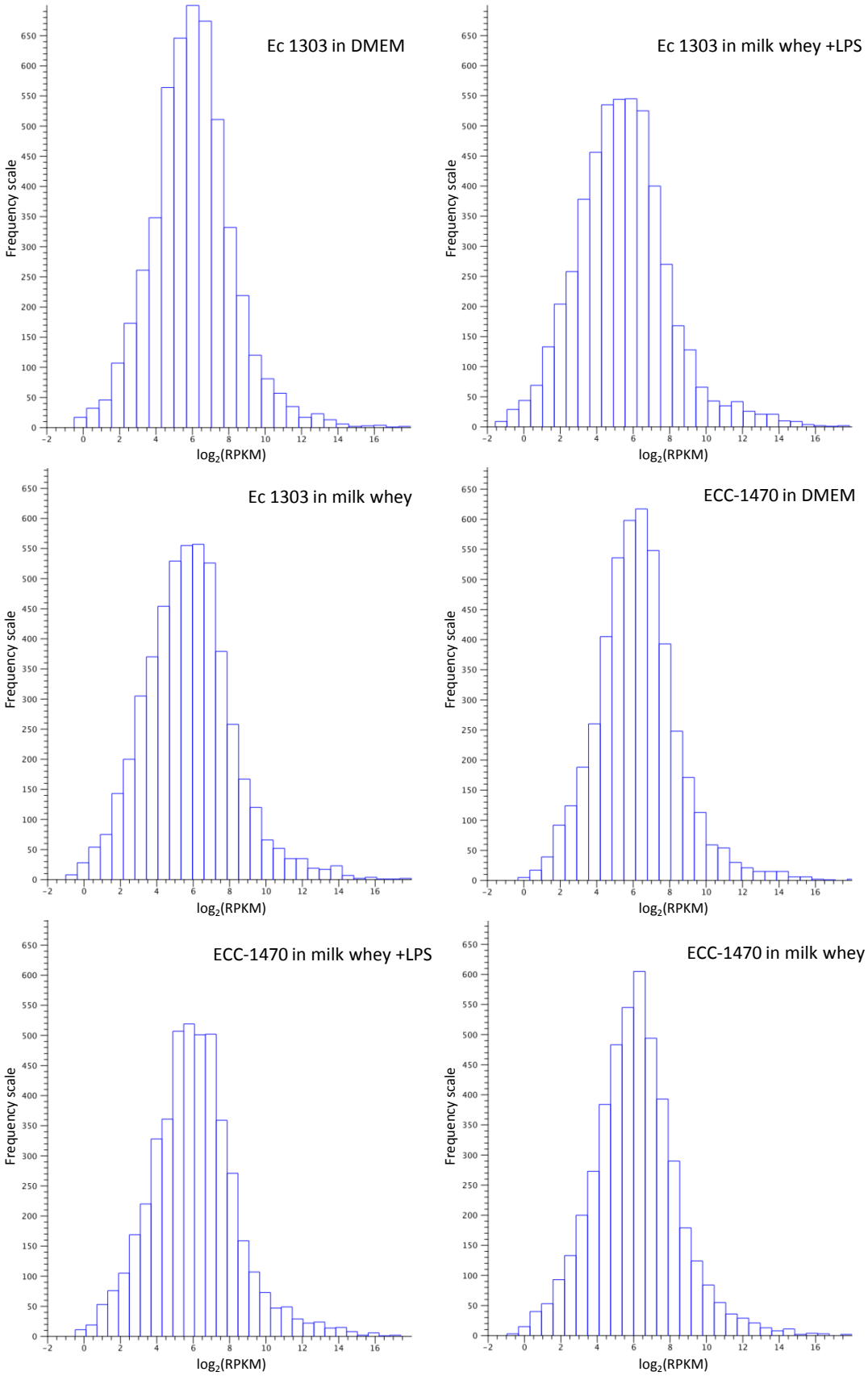

Figure 27: Distribution of expression values for annotated CDS of the genomes of *E. coli* strains 1303 and ECC-1470. Histograms display log_2-transformed RPKM expression values that show a normal distribution in all of the six samples.

According to **Figure 27**, the overall level of gene expression was similar under the different conditions (DMEM, milk…etc.), and only a small fraction of CDSs exhibited marked deviations of their expression level. For further analyses, the normalization according to sample size was, nevertheless, indispensable.

V.6.3. Gene expression of *E. coli* strain 1303 and ECC-1470 in milk whey

From the histograms it cannot unambiguously be observed whether the number of genes that are highly or even not expressed under the different growth conditions was similar or not. Thus, a textual output was generated with gene expression categorized according to Wickramasinghe et al., 2012.

Table 14 groups the genes of *E. coli* strains 1303 and ECC-1470 according to their expression level upon growth in DMEM, milk whey and milk whey after LPS challenge. The annotated reference genome of *E. coli* strain 1303 comprises 5,155 CDSs. while the genome of *E. coli* ECC-1470 includes 4,703 annotated CDSs. The 22 genes coding for rRNAs were removed from further analyses as stated before. Thus, a total of 5,133 annotated CDSs of *E. coli* strain 1303 and a total of 4,681 annotated CDS of strain ECC-1470 remained. For 2.1 to 3.0 % of the annotated CDSs per genome no reads could be mapped and they were thus defined as “non-expressed genes”. In summary,

Table 14 shows that the ratio of the total expressed genes and non-expressed genes between *E. coli* strains 1303 and ECC-1470 was quite similar.

Table 14: Grouping of genes of *E. coli* strains 1303 and ECC-1470 according to their expression level in DMEM, milk whey and milk whey upon LPS challenge. Expression values are indicated as RPKM.

Category	Strain	Condition		
		DMEM	Milk whey	Milk whey +LPS
Highly expressed genes (> 500 RPKM)	Ec 1303	400 †[8.0%]	417 †[8.3%]	372 †[7.4%]
	ECC-1470	427 †[9.3%]	435 †[9.5%]	419 †[9.1%]
Medium expressed genes (≥ 10 RPKM to 500 RPKM)	Ec 1303	4,089 †[81.7%]	3,826 †[76.5%]	3,667 †[73.3%]
	ECC-1470	3,755 †[82.0%]	3,662 †[79.9%]	3,626 †[79.2%]
Lowly expressed genes (< 10 RPKM)	Ec 1303	644 †[12.9%]	890 †[17.8%]	1,094 †[21.9%]
	ECC-1470	499 †[10.9%]	584 †[12.7%]	636 †[13.9%]
Total expressed genes	Ec 1303	5,003 *[97.5%]	4,994 *[97.3%]	4,978 *[97.0%]
	ECC-1470	4,581 *[97.9%]	4,576 *[97.8%]	4,560 *[97.4%]
Non-expressed genes	Ec 1303	130 *[2.5%]	139 *[2.7%]	155 *[3.0%]
	ECC-1470	100 *[2.1%]	105 *[2.2%]	121 *[2.6%]

† % refers to total expressed genes as 100%
* % refers to total CDS per strain as 100%

Interestingly, *E. coli* strain 1303 exhibited less highly expressed and less medium expressed genes than strain ECC-1470 under all conditions tested. This is even more remarkable, because the reference genome of mastitis isolate *E. coli* 1303 comprises 452 CDS more than *E. coli* ECC-1470. In

return, this reveals an unproportional higher number of highly expressed genes in *E. coli* ECC-1470 relative to the genome size. This indicates a stronger transcriptional response of this strain to milk whey and milk whey +LPS. In contrast, strain ECC-1470 revealed significantly less weakly expressed genes than *E. coli* 1303 under all growth conditions. In both strains, the number of highly and medium expressed genes decreased upon growth in milk whey or milk whey +LPS samples relative to growth in DMEM. To the same extent, the fraction of weakly expressed genes increased in milk whey and in milk whey +LPS samples relative to the DMEM samples.

In order to not identify only the genes with the highest expression level in a single sample (data on compact disk: **Supplemental Tables S2-S7**), but to identify differentially expressed genes within different samples, the samples needed to be investigated by statistical experiments. As a prerequisite, the comparability of the samples had to be ensured.

Quantile normalization of original expression values is required for sample comparison

A different sequencing depth affects the total read count per transcript of a sample. Consequently, this will result in inaccurate determination of differentially expressed genes when expression is quantified by read counts per transcript. In order to quantitatively and comparatively examine the number of reads per CDS in each sample, normalized sets of expression values of the samples were required. Therefore, the read counts might be divided by the total number of mapped reads or its quantile. In the present study, the data were normalized for sample size by quantile normalization. In quantile normalization, the distribution of read counts per sample (sequencing depth) is matched to a reference distribution defined in terms of median counts across the samples to compare (Gupta et al., 2012; Bullard et al., 2010). As it is assumed that gene expression in both strains may differ under the three conditions tested, two-group experiments were conducted (e.g. DMEM IRT milk whey or DMEM IRT milk whey +LPS or milk whey IRT milk whey +LPS). Due to the lack of technical replicates of RNA-Seq data sets, both strains 1303 and ECC-1470 were considered as biological replicates. The Box-Whisker-plot shown in **Figure 28** depicts the pairwise distributions of the samples´ expression values at different conditions compared to each other. Although the samples´ median values were quite similar, their quartiles differed. This indicated a variation across the samples. The same also applied to the whiskers outside the upper and lower quartiles; indicating variability. Notably, the gene expression data sets of mastitis *E. coli* strains 1303 and ECC-1470 appeared quite similar in value distribution and variation when incubated in DMEM, but exhibited marked differences in the variation of values, when cDNA have been investigated which resulted from incubations in milk whey or milk whey +LPS.

While the distributions of RPKM values (**Figure 27**) were in some degree normalized by the transcript size, the non-normalized read count distributions (**Figure 28**, *before normalization*) indicated that normalization was required before the sample could be compared to each other. Especially the

sample representing the gene expression data set of *E. coli* ECC-1470 upon incubation in milk whey +LPS, required normalization. This could also be expected according to the comparably low fraction of mappable reads (**Table 12**), which in turn resulted from poor PHRED scores (**Figure 24**). After quantile normalization, none of the normalized sample distributions differed from those of the other samples. Consequently, none of the samples had to be excluded and thus comparability between samples in statistical experiments was ensured.

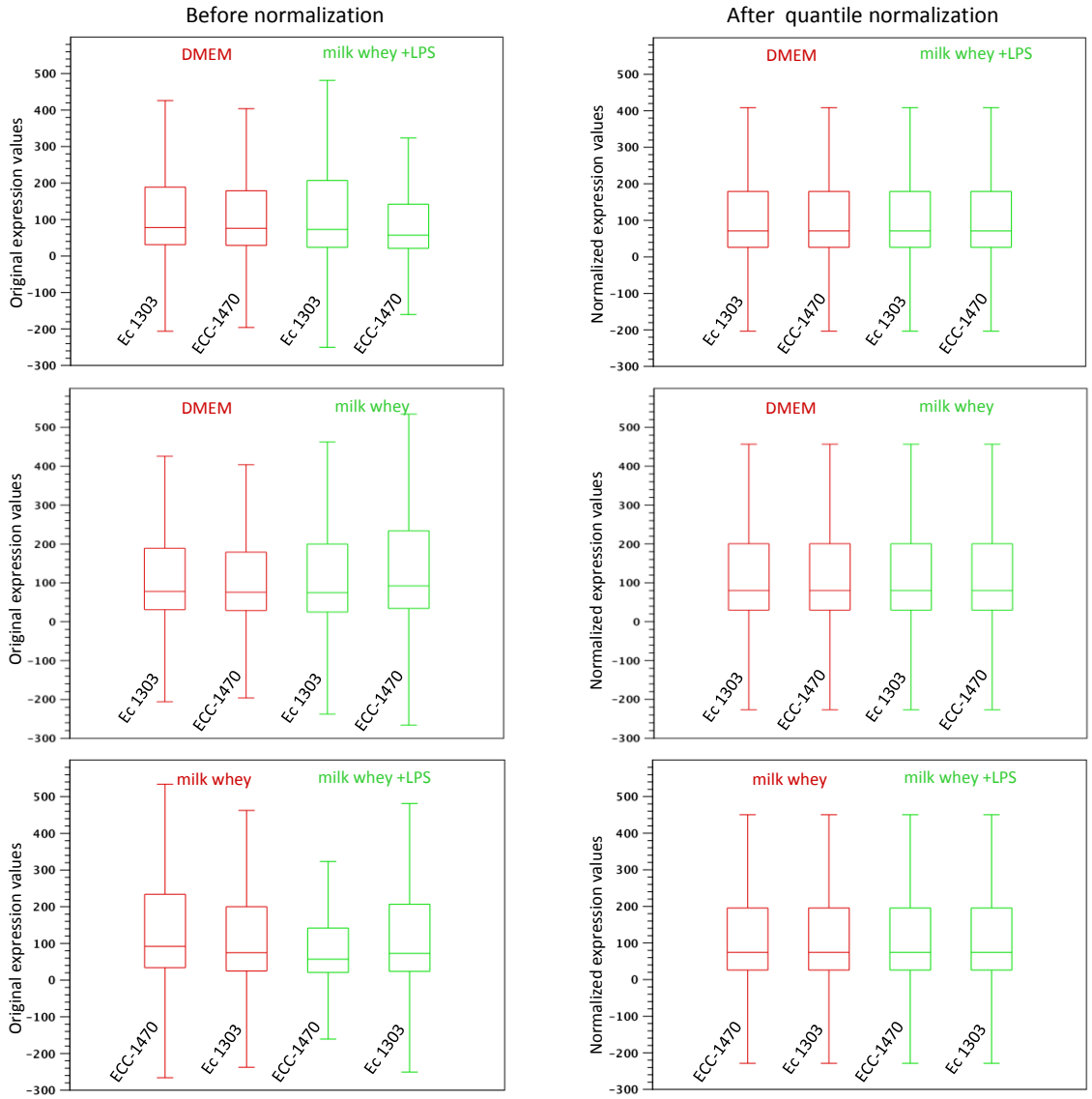

Figure 28: Box-Whisker-plot for two-group experiments with data sets from two samples of strain *E. coli* strains 1303 and ECC-1470 at different conditions, respectively. The left column depicts the data sets before normalization. The right column depicts the corresponding data sets after normalization.

V.6.4. Identification of differentially expressed genes in milk whey and milk whey +LPS

For RNA-Seq-based transcriptome analyses the quantification of expression values should be as accurate as possible and thus correct mapping of the reads is mandatory. While the RPKM-model was utilized to measure expression levels of different genes across different strains and conditions, the transcript length is not of importance when testing for differential gene expression. Here, the expression of the same gene is compared across samples (i.e. condition or strain), but not compared to other genes. Moreover, the RPKM model might result in biased estimates in determining differential gene expression across conditions tested due to the small portion of highly-expressed genes (Bullard et al., 2010). Thus, the transcript length should not be regarded for differential gene expression analysis, and instead of the RPKM-model, the total read count of each transcript/CDS was chosen to measure DEG.

Differentially expressed genes in milk whey and milk whey +LPS

Differential gene expression was discovered by performing two-group experiments using the bootstrapped Receiver Operating Characteristic algorithm (bROC) on quantile normalized read counts. The total read count of each gene was determined and the transcriptome of both *E. coli* strains 1303 and ECC-1470 were treated as biological replicates per state/condition. Sample data from both isolates at the corresponding condition were used for bROC analysis. Following this, the data were $\log_2$–transformed and unity shifted (+1), because the data included null values as well. The transformed read counts for both isolates cultivated in either milk whey or LPS-challenged milk whey were compared to the transformed read counts measured upon incubation in DMEM (or milk whey) in order to determine the $\log_2$-fold change of gene expression. Genes with a discrimination score of CONF > 0.95 were considered to be differentially expressed (**supplemental Tables S8-S10** on compact disc). As depicted in **Figure 29**, both mastitis *E. coli* strains 1303 and ECC-1470 exhibited deregulated genes when incubated in either milk whey or milk whey from a cow´s udder challenged with LPS relative to incubation in DMEM (or milk whey). Individual numbers of up- and down-regulated genes in both strains at the respective conditions are shown.

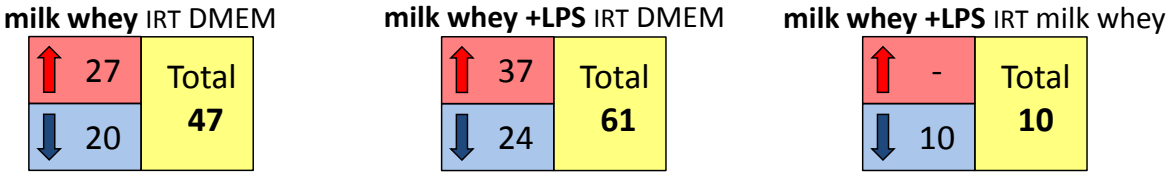

Figure 29: Deregulated genes in both *E. coli* strains 1303 and ECC-1470 after 1 h incubation at 37°C in: i) milk whey relative to DMEM cell culture medium, ii)) LPS-challenged milk whey relative to DMEM cell culture medium, and iii) LPS-challenged milk whey relative to milk whey. The diagram shows the numbers of significantly up- (red) and down- (blue) regulated genes (CONF > 0.95).

When incubated in milk whey from a cow´s udder challenged with LPS, both mastitis strains commonly exhibited an increased number of significantly deregulated genes relative to milk whey from an udder-healthy cow or to DMEM cell culture medium. This strongly suggests a specific response to components that are present in milk whey after contact to bacterial LPS.

In order to investigate which genes were deregulated in one condition as well as in another condition, the Venn algorithm was utilized. Therefore, the number of differentially expressed genes of mastitis strains *E. coli* 1303 and ECC-1470 at the respective conditions was visualized in a Venn diagram shown in **Figure 30**. A considerable number of genes were commonly deregulated in both strains, but not necessarily in response to the same condition as indicated by the overlapping areas (intersections) of three samples. Of approximately 5,155 CDS of *E. coli* 1303 and approximately 4,703 CDS of ECC-1470, the Venn algorithm identified a total of 88 different genes that were simultaneously deregulated in both strains after incubation in either milk whey or milk whey +LPS relative to DMEM. The particular intersections demonstrate, however, a transcriptional response by the same genes suggesting that the very same processes were affected in both strains though it is not possible to follow either up or down regulation in this Venn diagram. 18 genes were deregulated in milk whey as well as in milk whey +LPS. The number of genes exclusively deregulated in milk whey +LPS relative to DMEM (40 genes) was twofold higher than the number exclusively deregulated in milk whey +LPS in reference to DMEM (20 genes). No gene was detected to be uniquely deregulated in milk whey +LPS relative to milk whey while 10 genes were also deregulated in either milk whey or milk whey +LPS relative to DMEM. It is suggested, that the response to components, which are present or increased in milk whey after the cow´s contact to LPS (e.g. defensins or iron-binding proteins), resulted in a marked response of the gene expression levels in both mastitis *E. coli* strains relative to cell culture medium compared to milk whey without LPS challenge.

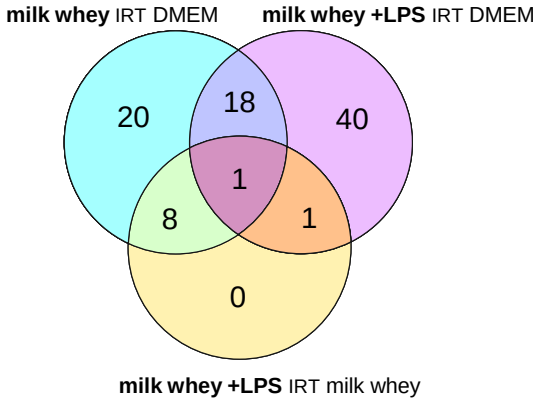

Figure 30: Impact of DMEM cell culture medium, milk whey or milk whey + LPS on gene expression of mastitis *E. coli* isolates as determined by RNA-Seq. The Venn diagram shows the numbers of significantly deregulated genes in *E. coli* strains 1303 and ECC-1470 upon incubation in milk whey or milk whey +LPS in relation to (IRT) DMEM cell culture medium/milk whey (CONF > 0.95). The numbers displayed in circles indicate the number of genes shared between the overlapping areas, which represent the sample-specific incubation medium.

If only the LPS challenge would have markedly affected deregulation of genes in response to milk whey, one would have identified genes deregulated uniquely in milk whey +LPS relative to milk whey as well (**Figure 30**).

In addition to the visual output of the Venn algorithm, it is also possible to investigate whether the 88 different genes were up- or down-regulated in milk whey or milk whey +LPS, respectively. Therefore, a corresponding textual output is given in **Table 15** showing which genes were (i) deregulated at distinct conditions or (ii) uniquely deregulated under a certain condition. Significantly deregulated genes (CONF >0.95) are listed according to their presence in each of the samples. Their corresponding fold log_2-fold change is indicated in either red (up-regulated) or blue (down-regulated).

While the Venn diagram's central intersection **Figure 30** revealed only one gene that was diversely deregulated between all conditions, it can now be identified as the *artJ* gene encoding the ABC transporter arginine binding protein 1. According to **Table 15** the log_2-fold change of *artJ* revealed an approximately twofold higher up-regulation in milk whey compared to its up-regulation in milk whey +LPS. This is corroborated by the negative ratio of the expression levels in milk whey +LPS in relation to milk whey. Besides *artJ*, nine other genes were repressed in milk whey +LPS relative to milk whey only. One of these particular genes was *iscR*, which was down-regulated not only in milk whey +LPS relative to milk whey but also in milk whey +LPS relative to DMEM. The 18 genes deregulated in milk whey and milk whey +LPS showed a consistent expression pattern in that they were either uniquely up- or down- but not differently regulated. These genes can be considered as deregulated in response to components of milk whey rather than components appearing or increasing after pathogen recognition. In contrast, eight genes were up-regulated in milk whey relative to DMEM, but were down-regulated in milk whey +LPS relative to milk whey. These genes are suggested to be especially useful in milk whey, which does not contain any host factors as a response to pathogen recognition (e.g. defensins or iron-binding proteins). It can be speculated that milk whey from an udder healthy cow still offers sufficient conditions for bacterial multiplication while milk whey from a cow´s udder challenged with LPS represents more aggressive growth conditions. Consequently, a normal response of the bacteria may be inhibited and instead bacterial countermeasure may be induced.

In summary it can be stated that both mastitis strains commonly deregulated a number of genes when they were incubated either in milk whey or in milk whey +LPS. These genes might represent specific bacterial responses to milk whey comprising e.g., antimicrobial components and these components increased upon LPS challenge. It can be hypothesized that at least some of the differentially expressed genes identified might be involved in the pathogenesis caused by the transient mastitis strain 1303 and persistent mastitis isolate ECC-1470.

Table 15: Deregulated genes of mastitic *E. coli* in milk whey or milk whey +LPS.

Sample / Intersection	Gene	Locus tags Ec 1303 / ECC-1470		Annotations - Protein product	Fold change Sample 1	Sample 2	Sample 3
Sample 1: milk whey IRT DMEM **Sample 2**: milk whey +LPS IRT DMEM; **Sample 3**: milk whey +LPS IRT milk whey	***artJ***	EPE_c00350	ECN_c44210	ABC transporter arginine-binding protein 1	5.05	2.70	-2.73
Sample 1: milk whey IRT DMEM **Sample 2:** milk whey +LPS IRT DMEM 18 genes total	***bioB***	EPE_c01290	ECN_c45060	Biotin synthase	-4.09	-4.15	no DE
	citF	EPE_c02920	ECN_c00830	citrate lyase, citrate-ACP transferase (alpha) subunit	3.41	4.71	no DE
	ecnB	EPE_c12150	ECN_c10250	entericidin B membrane lipoprotein	-2.69	-3.83	no DE
	entC	EPE_c03140	ECN_c01060	Isochorismate synthase entC	-3.87	-5.41	no DE
	entE	EPE_c03130	ECN_c01050	2, 3-dihydroxybenzoate-AMP ligase component of enterobactin synthase complex	-3.70	-3.69	no DE
	entF	EPE_c03210	ECN_c01130	Enterobactin synthase component F	-2.93	-2.99	no DE
	fes	EPE_c03230	ECN_c01150	enterobactin/ferric enterobactin esterase	-3.51	-3.94	no DE
	fhuF	EPE_c10050	ECN_c07930	ferric iron reductase involved in ferric hydroximate transport	-2.73	-4.39	no DE
	fruK	EPE_c33350	ECN_c30700	1-phosphofructokinase	-2.45	-2.27	no DE
	glnH	EPE_c00890	ECN_c44690	Glutamine-binding periplasmic protein	4.09	3.74	no DE
	glnP	EPE_c00900	ECN_c44700	Glutamine transport system permease protein glnP	3.14	3.77	no DE
	gltI	EPE_c02530	ECN_c00440	Glutamate/aspartate periplasmic-binding protein	3.08	2.85	no DE
	lipA	EPE_c02800	ECN_c00710	Lipoyl synthase	2.48	2.25	no DE
	mntH	EPE_c31230	ECN_c28450	manganese/divalent cation transporter	-2.73	-3.32	no DE
	nrdH	EPE_c28740	ECN_c25760	Hydrogen donor for NrdEF electron transport system	-3.43	-5.29	no DE
	rmf	EPE_c47870	ECN_c43280	Ribosome modulation factor	-3.47	-4.74	no DE
	yhaO	EPE_c23830	ECN_c20770	Inner membrane transport protein YhaO	-4.43	-4.11	no DE
	yncE	EPE_c41690	ECN_c38770	hypothetical protein YncE	-3.15	-3.87	no DE

Indicated "Fold change" refers to bROC analysis $\log_2$-fold change (CONF > 0.95). Significantly deregulated genes are indicated red (up) and blue (down).
Fold change of **1)** milk whey IRT DMEM, **2)** milk whey +LPS IRT DMEM, or **3)** milk whey +LPS IRT milk whey.

Sample / Intersection	Gene	Locus tags Ec 1303 / ECC-1470		Annotations - Protein product	Fold change Sample 1	Sample 2	Sample 3
Sample 1: milk whey IRT DMEM **Sample 3:** milk whey +LPS IRT milk whey 8 genes total	***argC***	EPE_c14200	ECN_c12200	N-acetyl-gamma-glutamyl-phosphate reductase	4.39	no DE	-3.66
	argI	EPE_c11060	ECN_c09170	Ornithine carbamoyltransferase	5.14	no DE	-3.39
	cysH	EPE_c27860	ECN_c24810	Phosphoadenosine phosphosulfate reductase	3.13	no DE	-3.37
	cysJ	EPE_c27840	ECN_c24790	Sulfite reductase [NADPH] flavoprotein alpha-component	4.06	no DE	-4.42
	cysP	EPE_c30910	ECN_c28160	Thiosulfate-binding protein	4.11	no DE	-4.25
	cysU	EPE_c30920	ECN_c28170	sulfate/thiosulfate transporter subunit	5.52	no DE	-4.16
	iaaA	EPE_c00710	ECN_c44520	Isoaspartyl peptidase	2.87	no DE	-3.08
	ydjN	EPE_c38090	ECN_c35610	Uncharacterized symporter ydjN	2.93	no DE	-3.31
Sample 2: milk whey +LPS IRT DMEM **Sample 3:** milk whey +LPS IRT milk whey	***iscR***	EPE_c29880	ECN_c27130	DNA-binding transcriptional repressor	no DE	-4.18	-2.77
Sample 1: milk whey IRT DMEM 20 genes total	***argT***	EPE_c31910	ECN_c29380	lysine/arginine/ornithine transporter subunit	2.83	no DE	no DE
	bioF	EPE_c01280	ECN_c45050	8-amino-7-oxononanoate synthase	-4.02	no DE	no DE
	citE	EPE_c02910	ECN_c00820	citrate lyase, citryl-ACP lyase (beta) subunit	4.43	no DE	no DE
	citX	EPE_c02930	ECN_c00840	Apo-citrate lyase phosphoribosyl-dephospho-CoA transferase	7.57	no DE	no DE
	cpxP	EPE_c14640	ECN_c12690	inhibitor of the cpx response / periplasmic adaptor protein	-2.39	no DE	no DE
	cysA	EPE_c30940	ECN_c28190	Sulfate/thiosulfate import ATP-binding protein CysA	2.91	no DE	no DE
	cysD	EPE_c27920	ECN_c24940	Sulfate adenylyltransferase subunit 2	2.58	no DE	no DE
	fepA	EPE_c03240	ECN_c01160	iron-enterobactin outer membrane transporter	-4.05	no DE	no DE
	gdhA	EPE_c37770	ECN_c35280	NADP-specific glutamate dehydrogenase	2.98	no DE	no DE
	hycA	EPE_c28210	ECN_c25250	regulator of the transcriptional regulator FhlA	3.51	no DE	no DE
	metF	EPE_c14370	ECN_c12370	5, 10-methylenetetrahydrofolate reductase	3.61	no DE	no DE
	nrdI	EPE_c28730	ECN_c25750	flavodoxin required for NrdEF cluster assembly	-3.91	no DE	no DE
	nuoA	EPE_c32130	ECN_c29600	NADH:ubiquinone oxidoreductase, membrane subunit A	2.55	no DE	no DE
	potF	EPE_c00410	ECN_c44270	Putrescine-binding periplasmic protein	3.59	no DE	no DE

Sample / Intersection	Gene	Locus tags Ec 1303 / ECC-1470		Annotations - Protein product	Fold change Sample 1	Sample 2	Sample 3
Sample 1: milk whey IRT DMEM 20 genes total	***sufA***	EPE_c39030	ECN_c36060	Fe-S cluster assembly protein	-3.35	no DE	no DE
	yagU	EPE_c06020	ECN_c04360	Inner membrane protein yagU	2.83	no DE	no DE
	ygeO	EPE_c26830	ECN_c23580	type III secretion apparatus system protein	-3.40	no DE	no DE
	yjiH	EPE_c10630	ECN_c08310	hypothetical protein yjiH	3.59	no DE	no DE
	yjjZ	EPE_c10040	ECN_c07920	hypothetical protein	-4.13	no DE	no DE
	ylaC	EPE_c04300	ECN_c02670	Inner membrane protein ylaC	3.84	no DE	no DE
Sample 2: milk whey +LPS IRT DMEM 40 genes total	***aceE***	EPE_c07950	ECN_c06390	pyruvate dehydrogenase, decarboxylase component E1	no DE	1.88	no DE
	argB	EPE_c14190	ECN_c12190	Acetylglutamate kinase	no DE	-2.40	no DE
	artI	EPE_c00320	ECN_c44180	Putative ABC transporter arginine-binding protein 2	no DE	2.47	no DE
	cirA	EPE_c33480	ECN_c30830	catecholate siderophore receptor CirA, colicin I receptor	no DE	-2.42	no DE
	citC	EPE_c02890	ECN_c00800	[Citrate [pro-3S]-lyase] ligase	no DE	4.04	no DE
	citG	EPE_c02940	ECN_c00850	2-(5''-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase	no DE	3.70	no DE
	codA	EPE_c05470	ECN_c03820	Cytosine deaminase	no DE	2.28	no DE
	cutC	EPE_c36540	ECN_c34020	Copper homeostasis protein CutC	no DE	-1.79	no DE
	fecI	EPE_c25370	ECN_c08760	KpLE2 phage-like element / RNA polymerase, sigma 19 factor	no DE	-3.55	no DE
	fecR	EPE_c25380	ECN_c08770	KpLE2 phage-like element / transmembrane signal transducer for ferric citrate transport	no DE	-2.61	no DE
	fhuA	EPE_c07570	ECN_c06030	Ferrichrome-iron receptor	no DE	-1.85	no DE
	flhC	EPE_c36370	ECN_c33850	DNA-binding transcriptional dual regulator with FlhD	no DE	2.61	no DE
	frdD	EPE_c12110	ECN_c10210	fumarate reductase (anaerobic), membrane anchor subunit	no DE	2.32	no DE
	ftnA	EPE_c36250	ECN_c33730	ferritin iron storage protein (cytoplasmic)	no DE	3.37	no DE
	glnQ	EPE_c00910	ECN_c44710	Glutamine transport ATP-binding protein GlnQ	no DE	3.49	no DE
	gpmA	EPE_c01510	ECN_c45270	2, 3-bisphosphoglycerate-dependent phosphoglycerate mutase	no DE	-2.74	no DE
	osmC	EPE_c41380	ECN_c38470	Lipoyl-dependent Cys-based peroxidase, hydroperoxide resistance	no DE	-2.97	no DE

Sample / Intersection	Gene	Locus tags Ec 1303 / ECC-1470		Annotations - Protein product	Fold change Sample 1	Sample 2	Sample 3
Sample 2: milk whey +LPS IRT DMEM 40 genes total	***pepE***	EPE_c13530	ECN_c11560	(alpha)-aspartyl dipeptidase	no DE	3.07	no DE
	potD	EPE_c45310	ECN_c41570	polyamine transporter subunit / periplasmic-binding component of ABC superfamily	no DE	2.28	no DE
	ptsG	EPE_c45530	ECN_c41790	fused glucose-specific PTS enzymes: IIB component / IIC component	no DE	2.34	no DE
	rplB	EPE_c21710	ECN_c18670	50S ribosomal subunit protein L2	no DE	3.10	no DE
	rplD	EPE_c21690	ECN_c18650	50S ribosomal subunit protein L4	no DE	3.20	no DE
	rplF	EPE_c21830	ECN_c18790	50S ribosomal subunit protein L6	no DE	2.48	no DE
	rplI	EPE_c11590	ECN_c09700	50S ribosomal protein L9	no DE	2.73	no DE
	rplR	EPE_c21840	ECN_c18800	50S ribosomal subunit protein L18	no DE	2.60	no DE
	rplS	EPE_c29100	ECN_c26380	50S ribosomal subunit protein L19	no DE	2.67	no DE
	rplV	EPE_c21730	ECN_c18690	50S ribosomal subunit protein L22	no DE	2.34	no DE
	rplW	EPE_c21700	ECN_c18660	50S ribosomal subunit protein L23	no DE	3.42	no DE
	rpmC	EPE_c21760	ECN_c18720	50S ribosomal subunit protein L29	no DE	2.76	no DE
	rpmI	EPE_c38220	ECN_c35740	50S ribosomal subunit protein L35	no DE	2.12	no DE
	rpsE	EPE_c21850	ECN_c18810	30S ribosomal subunit protein S5	no DE	2.45	no DE
	rpsG	EPE_c21450	ECN_c18570	30S ribosomal protein S7	no DE	2.81	no DE
	rpsI	EPE_c22600	ECN_c19530	30S ribosomal subunit protein S9	no DE	2.21	no DE
	rpsN	EPE_c21810	ECN_c18770	30S ribosomal subunit protein S14	no DE	2.74	no DE
	rpsR	EPE_c11600	ECN_c09710	30S ribosomal protein S18	no DE	2.92	no DE
	rpsS	EPE_c21720	ECN_c18680	30S ribosomal subunit protein S19	no DE	2.27	no DE
	tsx	EPE_c04770	ECN_c03160	nucleoside channel, receptor of phage T6 and colicin K	no DE	2.24	no DE
	ybgE	EPE_c01730	ECN_c45470	hypothetical protein ybgE	no DE	2.67	no DE
	ydiY	EPE_c38160	ECN_c35680	predicted outer membrane protein, acid-inducible	no DE	2.62	no DE
	yjbJ	EPE_c13260	ECN_c11260	UPF0337 protein yjbJ	no DE	-2.60	no DE

IRT in reference to

V.6.5. Commonly deregulated genes in response to milk whey

Hierarchical clustering of deregulated genes

In order to identify commonly deregulated genes of both *E. coli* mastitis strains 1303 and ECC-1470 when incubated in either milk whey or milk whey +LPS or DMEM, hierarchical cluster analyses of the identified deregulated genes were performed. Genes, which exhibited the same or a similar expression profile, were grouped together. The significantly deregulated genes after one hour incubation in the respective medium differed in their expression levels across both mastitis isolates (also visible in **Table 15** on page 130). This is indicating a distinct transcriptional response of the two different strains.

The isolates´ transcriptional response to milk whey relative to DMEM resulted in commonly deregulated genes and revealed five subclusters shown in **Figure 31**. Most genes of *E. coli* strains 1303 and ECC-1470 were not only commonly, but also evenly regulated after one hour in DMEM. In contrast, in milk whey relative to DMEM most deregulated genes showed distinct expression levels between isolates although their regulation is following a common underlying trend (up or down).

Specifically, the first subcluster identified upon growth in milk whey includes down-regulated genes involved in iron acquisition (*fhuF*, *entF*, *mntH*, *fepA* and *yncE*) and *ecnB* encoding for the lipoprotein entericidin, which plays a role in programmed cell death under high osmolarity conditions. Down-regulation of iron-associated genes was further supported by the second subcluster of deregulated genes include many genes coding for components of the enterobactin synthase complex (*entC*, *entE* and *fes*). It is somehow notable but not surprising that *entF* is allocated to subcluster one whereas the fold total expression level is still much higher in comparison to *entC* and *entE*.

Of the three remaining subclusters, two included a majority of up-regulated genes involved in amino acid metabolism as well as the inner membrane proteins *ylaC* and *yjiH* as well as the genes *citE and citF* whose gene products are required for citrate utilization. Interestingly, *potF*, which is coding for a putrescine-binding periplasmic protein was commonly up-regulated as well. It has been previously discussed that amino acid synthesis, especially that of glutamate, and putrescine release are signals of hyperosmotic stress (Koegel, 2008). We identified positively deregulated genes associated with glutamine/glutamate synthesis as well (*glnH*, *glnP* and *gltI*) corroborating that the common deregulation of *potF* is not a coincidence. The fifth and last subcluster of commonly deregulated genes in milk whey relative to DMEM included mainly genes of comparably high expression levels. Of these, the most expressed genes were *artJ*, *glnH* and *metF* associated with arginine, glutamate and methionine synthesis while expression of *gdhA* and *iaaA* was also up-regulated and required for amino acid transport and processing. Furthermore, genes whose encoded proteins are involved in energy metabolism (*lipA*) as well as membrane/associated proteins (*nuoA*, *yagU* and *ydjN*) showed a common intense expression.

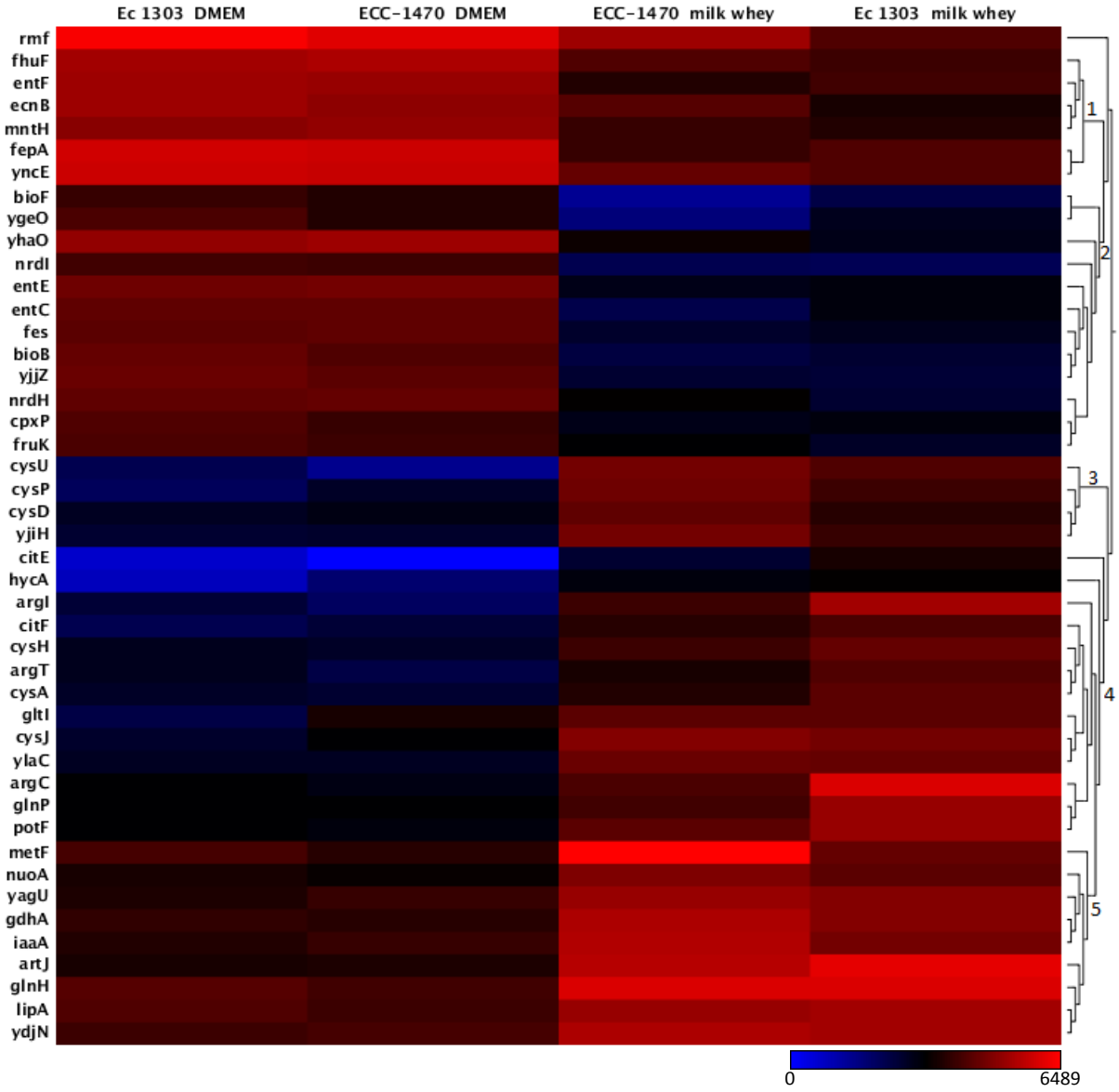

Figure 31: Differential expression of *E. coli* genes in DMEM and milk whey.
Hierarchical cluster plot showing differentially expressed genes of mastitis *E. coli* strains 1303 and ECC-1470 upon 1 h incubation at 37°C in DMEM cell culture medium in comparison to milk whey from an udder-healthy cow. Each bar represents one gene and its normalized expression level is colored according to the color bar. The datasets for each strain and condition are obtained from bROC analysis to discover differentially expressed genes. Genes with CONF > 0.95 are considered to be differentially expressed.

While the individual expression levels of commonly deregulated genes differed in milk whey, the transcriptional response to milk whey from an LPS-challenged cow was more uniform in both strains (**Figure 32**). The genes deregulated in *E. coli* strains 1303 and ECC-1470 were not only commonly deregulated, but revealed also similar expression levels in both strains when incubated one hour in either DMEM or milk whey +LPS. The average expression level of the genes in milk whey +LPS was comparably higher relative to that observed in pure milk whey (**Figure 31**). Commonly deregulated genes were allocated to two main clusters and various subclusters.

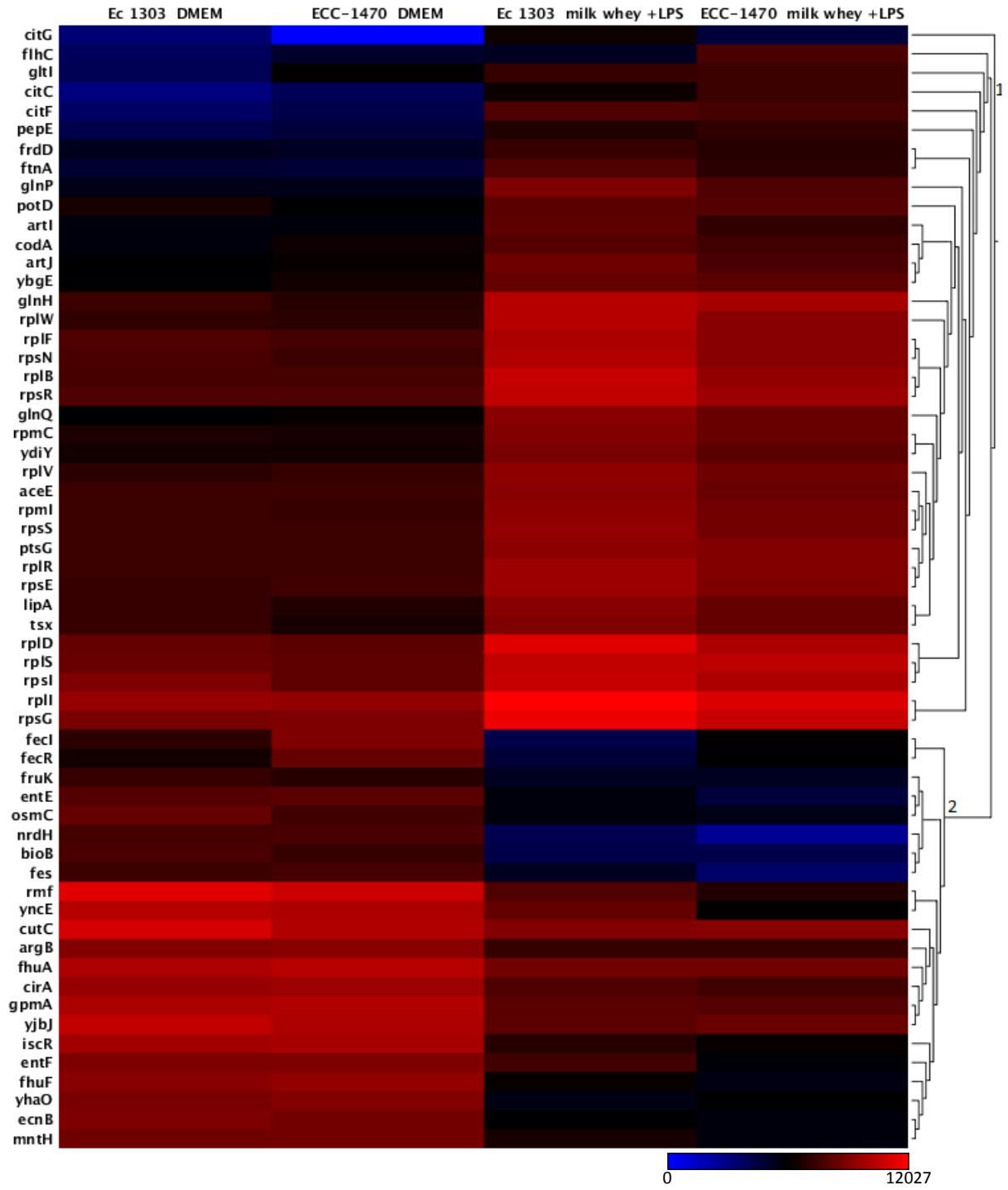

Figure 32: Differential expression of *E. coli* genes in DMEM and milk whey +LPS.
Hierarchical cluster plot showing differentially expressed genes of mastitis *E. coli* strains 1303 and ECC-1470 upon 1 h incubation at 37°C in DMEM cell culture medium in comparison to milk whey from a cow´s udder challenged with LPS. Each bar represents one gene and its normalized expression level is colored according to the color bar. The datasets for each strain and condition are obtained from bROC analysis to discover differentially expressed genes. Genes with CONF > 0.95 are considered to be differentially expressed.

Highest expression has been identified within the subclusters comprising genes coding for components of the 50S and 30S ribosomal subunits (*rpl*, *rpm* and *rps*). Further commonly deregulated genes among these subclusters included the hypothetical protein-encoding gene *ydiY*

and the genes *aceE*, *lipA* and *ptsG* required for energy metabolism. Although not really clustered, we again identified repression of gene expression associated with amino acid synthesis (*argB*, *artJ*, *glnH*, *glnP*, *gltI* and *pepE*).

Down-regulated genes involved in iron acquisition clustered in the second main cluster (*bioB*, *cirA*, *entE*, *entF*, *fecI*, *fecR*, *fes*, *fhuA*, *fhuF* and *mntH*). In contrast, *ftnA* encoding a cytoplasmic iron storage protein was not commonly down-regulated, but significantly up-regulated. The gene *ftnA* thereby represents the only gene associated to iron processing, that was exclusively up-regulated in milk whey +LPS, but not in pure milk whey. The cluster analysis also revealed a common up-regulation of genes (*citC*, *citF* and *citG*) involved in citrate utilization. Up-regulation of these genes was already identified in milk whey, but clustering occurred only when deregulated milk whey +LPS.

In order to examine whether genes were commonly deregulated in milk whey +LPS relative to milk whey, a third heat map depicts the deregulated genes between these two conditions (**Figure 33**). As much as both mastitis isolates displayed distinct transcriptional levels of the deregulated genes when incubated in milk whey (**Figure 31**), the transcriptional pattern of differentially expressed genes between milk whey +LPS and milk whey was highly erratic. Nevertheless, the cluster analysis revealed two subclusters of commonly deregulated genes depicting the general trend of either up- or down-regulation of these genes and whether their expression was comparably high or low.

The first (blue) subcluster reflects mainly down-regulated genes involved in amino acid synthesis and –utilization (**Figure 33**). Notably, *artJ* encoding the arginine binding protein 1 was also negatively deregulated but was not assigned to the same cluster, but to cluster two instead. This may be due to the still much higher expression level in milk whey +LPS than in milk whey despite the -2.7 fold change relative to milk whey (also noted in **Table 15** on page 130).

The second cluster shows a down-regulation of the peptidase encoding gene *iaaA* reflecting catabolic amino acid processes. This might *vice versa* support the notion of enhanced amino acid biosynthesis as suggested by increased gene expression of relevant genes. Cluster 2 comprised the DNA-binding transcriptional repressor gene *iscR* but also *ydjN* coding for an uncharacterized symporter protein. Both genes were negatively deregulated.

The hierarchical cluster analysis of the transcriptional response of *E. coli* strains 1303 and ECC-1470 to DMEM, milk whey and milk whey +LPS revealed not only commonly deregulated genes relative to DMEM, but also sample-specific differences in gene expression patterns. Deregulated genes that were clustered indicate a common deregulation of genes associated with amino acid metabolism and iron utilization.

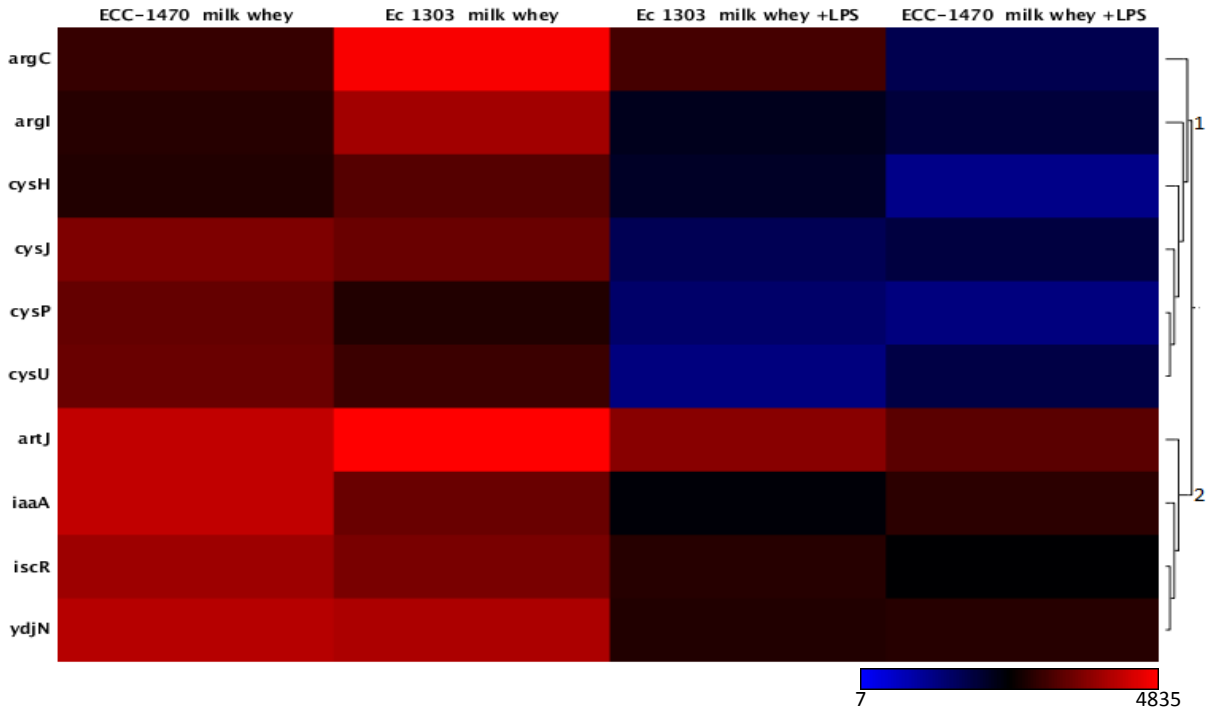

Figure 33: Differential expression of *E. coli* genes in milk whey and milk whey +LPS.
Hierarchical cluster plot showing differential expression of genes of mastitis *E. coli* strains 1303 and ECC-1470 upon 1 h incubation at 37°C in milk whey from an udder-healthy cow in comparison to milk whey from a cow´s udder challenged with LPS. Each bar represents one gene and its normalized expression level is coloured according to the colour bar. The datasets for each strain and condition are obtained from bROC analysis to discover differentially expressed genes. Genes with CONF > 0.95 are considered to be differentially expressed.

In contrast, genes associated with carbon utilization (e.g. citrate) or energy metabolism, change of growth phase (including programmed cell death), as well as genes encoding inner membrane proteins and hypothetical proteins have not been grouped together by hierarchical clustering. Genes associated with stress response (oxidative, osmotic or acidic), however, clustered together. The distinct high expression level of particular commonly deregulated genes (**Figure 31**, **Figure 33**) may reflect that both strains were not really biological replicates. Individually deregulated genes might rather indicate strain-specific properties than a common transcriptional response. Besides their distinct genome content, *E. coli* strains 1303 and ECC-1470 are diverse in that both isolates caused a different aetiological outcome of mastitis. It has been shown that they, nevertheless, employ a common transcriptional activity in response to milk whey. This has been depicted by the heat maps. Genes that were commonly deregulated indicate expression of the same or related cellular processes in response to growth in DMEM or milk whey or milk whey +LPS.

Functional classification of deregulated genes

In order to examine what cellular processes were reflected by the transcriptional response to milk whey and milk whey +LPS, we evaluated the differentially expressed genes according to their

functional classification. The deregulated genes were assigned to functional categories (**Figure 34**) based on the Clusters of Orthologous Genes (COG) designations as suggested by (Yoder-Himes et al., 2009). It is further suggested that the COG categories might reflect bacterial expression patterns in response to the environmental or bactericidal conditions in milk whey, or milk whey +LPS to countermeasure the cow´s immune response. The biggest fraction of genes deregulated under both conditions was assigned to category "Amino acid metabolism and transport" (E). Interestingly, differentially expressed genes representing "Translational associated functions" (J) were exclusively deregulated upon growth in milk whey +LPS. Besides categories C, G, J, K and M, the amount of deregulated genes was always higher when bacteria were incubated in milk whey than in milk whey +LPS. Thus, this suggests that bacterial growth in milk whey +LPS required more energy production and conversion as well as carbohydrate metabolism and transport than in milk whey.

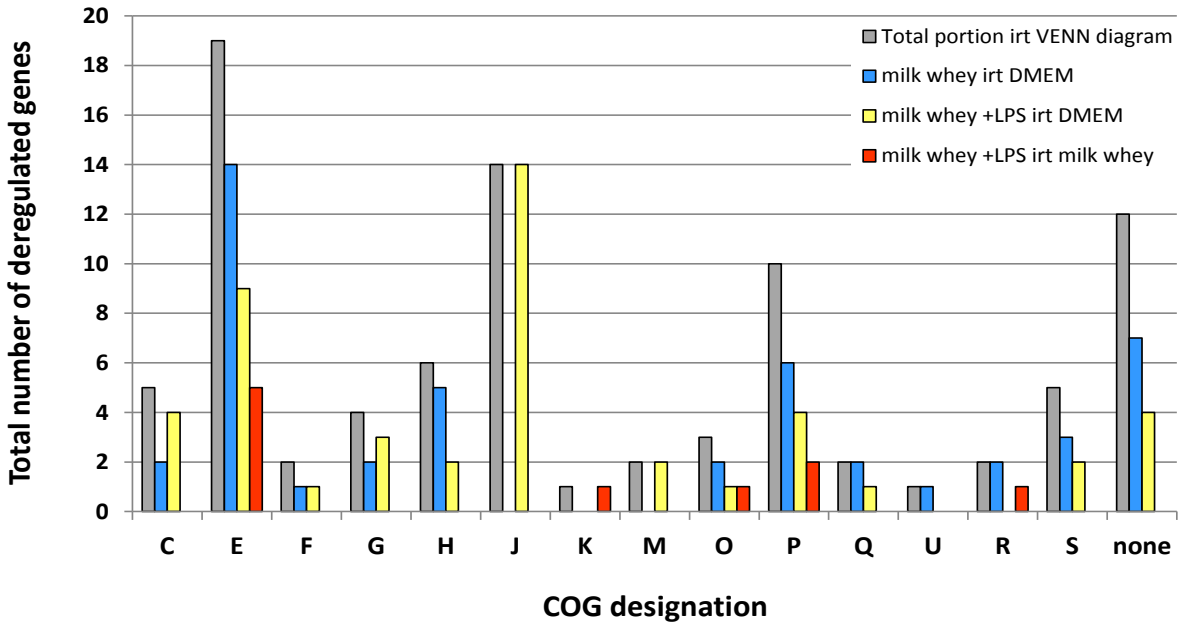

Figure 34: Functional classification of deregulated genes of *E. coli* mastitis isolates 1303 and 1470 in milk whey and milk whey +LPS. The numbers of genes belonging to the individual COG categories are depicted as total number of deregulated genes in the respective experimental set. Grey bars represent the total portion of the 88 different deregulated genes as revealed by the Venn diagram and may comprise genes deregulated in more than one of the experimental sets. COG designations are listed in **Table 16**.

Table 16: Clusters of Orthologous Genes COG classification

C	Energy production and conversion	M	Cell wall/membrane/envelop biogenesis
E	Amino acid metabolism and transport	O	Post-transl. modification, protein turnover, chaperone funct.
F	Nucleotide metabolism and transport	P	Inorganic ion transport and metabolism
G	Carbohydrate metabolism and transport	Q	Secondary structure
H	Coenzyme metabolism	U	Intracellular trafficking and secretion
J	Translation	R	General functional prediction only
K	Transcription	S	Function unknown

V.6.6. The transcriptional response to milk whey and milk whey +LPS

Investigating the transcriptional response of mastitis *E. coli* isolates 1303 and ECC-1470 to milk whey and milk whey +LSP one could expect diverse adaptations to the conditions conveyed by these media in contrast to a less hostile environment. The functional classification based on COG designations defined cellular processes, which were affected by growth under these conditions. However, specific cellular processes, which could be interpreted as a response to milk whey or milk whey + LPS, could not be revealed by this. In order to gain insight into the particular processes involved, it was necessary to categorize the identified deregulated genes according to their biological role. In **Table 17**, the genes that were deregulated in milk whey and milk whey +LPS are listed according to (i) their gene symbol, (ii) the cellular compartment(s) of the encoded protein and (iii) their molecular function. The same color code indicates the assigned categories. The correlation of up- or down-regulation of the expression of genes with their function and cellular localization made it possible to gain a surprisingly detailed view of what actually occurred in the mastitis *E. coli* isolates 1303 and ECC-1470 when grown in milk whey or in milk whey +LSP.

Iron utilization

Bacterial determinants that were categorized to be involved in iron uptake and -utilization represented the major fraction of deregulated genes. It has been suggested before, that mastitis isolates need to cope with harsh nutritional conditions in mammary secretions like iron shortage mediated by lactoferrin (Latorre et al., 2010). Surprisingly, both strains exhibited a marked repression of genes involved in iron uptake and utilization (**Table 17**). Among them, genes of the siderophore gene clusters encoding the components of the enterobactin (***ent***) and the ferric di-citrate transport system (***fec***) as well as siderophore receptor gene ***cirA*** were determined. Especially, we identified negatively deregulated regulatory determinants of the ***fec*** siderophore cluster. *fecI* encodes a specialized sigma factor and *fecR* is known to encode the regulator of the *fec* operon (Enz et al., 1995). Due to our assumption, that iron acquisition might be advantageous for survival and growth in mammary secretions, especially in presence of lactoferrin, it was rather surprising to find iron uptake systems to be down-regulated in *E. coli* in milk whey or in milk whey +LPS. Notably, ***ftnA*** which encodes a ferritin iron storage protein was the only gene associated with iron utilization that was positively deregulated in milk whey +LPS, but was not deregulated in milk whey relative to DMEM. At least, *ftnA* expression might indicate an increase in iron scavenging host proteins dissolved in mammary secretions when LPS has been sensed. *De facto*, we state that iron availability did not represent a key factor for *E. coli* mastitis isolates in mammary secretions at this stage. The expression of genes involved in iron utilization appeared to be of rather minor importance during the initial growth of *E. coli* than at later stages of infection.

Table 17: Functional processes important for growth of *E. coli* genes in milk whey and milk whey +LPS as reflected by differential gene expression relative to growth in DMEM medium

Gene	Annotations - Protein product	Category	Compartment	1)	2)	3)
yhaO	transport protein Yha, AA/proton symporter	amino acid transport	inner membrane			
argB	acetylglutamate kinase	arginine biosynth.	cytosol			
argC	N-acetyl-gamma-glutamyl-phosphate reductase	arginine biosynth.	cytosol			
argI	ornithine carbamoyltransferase	arginine biosynth.	cytosol			
argT	lysine/arginine/ornithine transporter subunit	arginine biosynth.	perplasmic space			
artI	putative ABC transporter arginine-binding protein 2	arginine biosynth.	perplasmic space			
artJ	ABC transporter arginine-binding protein 1	arginine biosynth.	perplasmic space			
gdhA	NADP-specific glutamate dehydrogenase	glutamate biosynth.	cytosol			
glnQ	glutamine transport ATP-binding protein GlnQ	glutamine biosynth.	inner membrane			
glnP	glutamine transport system permease protein glnP	glutamine biosynth.	inner membrane			
glnH	glutamine-binding periplasmic protein	glutamine biosynth.	perplasmic space			
gltI	glutamate/aspartate periplasmic-binding protein	glutamine biosynth.	perplasmic space			
metF	5, 10-methylenetetrahydrofolate reductase	methionine biosynth.	cytosol			
iaaA	isoaspartyl peptidase	peptidase	cytosol			
pepE	(alpha)-aspartyl dipeptidase	peptidase	cytosol			
fruK	1-phosphofructokinase	energy metabolism	cytosol			
gpmA	2, 3-bisphosphoglycerate-dependent phosphoglycerate mutase	energy metabolism	cytosol			
lipA	lipoyl synthase, cofactor of pyruvate dehydrogenase	energy metabolism	cytosol			
aceE	pyruvate dehydrogenase, decarboxylase component E1	energy metabolism	cytosol, membrane			
frdD	fumarate reductase (anaerobic), membrane anchor subunit	energy metabolism	inner membrane			
ptsG	fused glucose-specific PTS enzymes: IIB component / IIC component	energy metabolism	inner membrane			
nuoA	NADH:ubiquinone oxidoreductase, membrane subunit A	energy metabolism	inner membrane			
citC	[citrate [pro-3S]-lyase] ligase	citrate utilization	cytosol			
citE	citrate lyase, citryl-ACP lyase (beta) subunit	citrate utilization	cytosol			
citF	citrate lyase, citrate-ACP transferase (alpha) subunit	citrate utilization	cytosol			
citG	2-(5''-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase	citrate utilization	cytosol			
citX	apo-citrate lyase phosphoribosyl-dephospho-CoA transferase	citrate utilization	cytosol			
ygeO	type III secretion apparatus system protein	virulence associated	inner membrane, outer membrane			
tsx	nucleoside channel, receptor of phage T6 and colicin K, porine	virulence associated	inner membrane, outer membrane			
rplB	50S ribosomal subunit protein L2	exponential growth	cytosol			
rplD	50S ribosomal subunit protein L4	exponential growth	cytosol			
rplF	50S ribosomal subunit protein L6	exponential growth	cytosol			
rplI	50S ribosomal protein L9	exponential growth	cytosol			
rplR	50S ribosomal subunit protein L18	exponential growth	cytosol			
rplS	50S ribosomal subunit protein L19	exponential growth	cytosol			
rplV	50S ribosomal subunit protein L22	exponential growth	cytosol			
rplW	50S ribosomal subunit protein L23	exponential growth	cytosol			
rpmC	50S ribosomal subunit protein L29	exponential growth	cytosol			
rpmI	50S ribosomal subunit protein L35	exponential growth	cytosol			
rpsE	30S ribosomal subunit protein S5	exponential growth	cytosol			
rpsG	30S ribosomal protein S7	exponential growth	cytosol			
rpsI	30S ribosomal subunit protein S9	exponential growth	cytosol			
rpsN	30S ribosomal subunit protein S14	exponential growth	cytosol			
rpsR	30S ribosomal protein S18	exponential growth	cytosol			
rpsS	30S ribosomal subunit protein S19	exponential growth	cytosol			

Up-regulation (red) or down-regulation (blue) of two-group comparison experiments of deregulated genes
1) milk whey IRT DMEM, **2)** milk whey +LPS IRT DMEM, or **3)** milk whey +LPS IRT milk whey.

Gene	Annotations - Protein product	Category	Compartment	1)	2)	3)
entE	2, 3-dihydroxybenzoate-AMP	iron acquisition	inner membrane, cytosol, membrane			
entE	ligase component of enterobactin synthase complex	iron acquisition	inner membrane, cytosol, membrane			
entF	enterobactin synthase component F	iron acquisition	inner membrane, cytosol, membrane			
fhuF	ferric iron reductase involved in ferric hydroximate transport	iron acquisition	inner membrane, cytosol, membrane			
bioB	biotin synthase	iron acquisition	cytosol			
bioF	8-amino-7-oxononanoate synthase	iron acquisition	cytosol			
fecI	KpLE2 phage-like element / RNA polymerase, sigma 19 factor	iron acquisition	cytosol			
fes	enterobactin/ferric enterobactin esterase	iron acquisition	cytosol			
sufA	Fe-S cluster assembly protein	iron acquisition	cytosol			
mntH	manganese/divalent cation transporter	iron acquisition	inner membrane			
cirA	catecholate siderophore receptor CirA, colicin I receptor	iron acquisition	inner membrane, outer membrane			
entC / fepF	isochorismate synthase entC	iron acquisition	inner membrane, cytosol, membrane			
fepA	iron-enterobactin outer membrane transporter	iron acquisition	outer membrane			
fhuA	ferrichrome-iron receptor	iron acquisition	outer membrane			
fecR	KpLE2 phage-like element / transmembrane signal transducer for ferric citrate transport	iron acquisition	perplasmic space			
yncE	protein YncE involved in iron acquisition	iron acquisition	perplasmic space			
ftnA	ferritin iron storage protein (cytoplasmic)	iron storage	cytosol			
cpxP	inhibitor of the cpx response / periplasmic adaptor protein	REGULATOR	periplasmic space, cytosol			
flhC	DNA-binding transcriptional dual regulator with FlhD	RGEULATOR	cytosol			
hycA	regulator of the transcriptional regulator FhlA	REGULATOR	cytosol			
iscR	DNA-binding transcriptional repressor of the iron sulfur cluster	REGULATOR	cytosol			
rmf	ribosome modulation factor	REGULATOR	cytosol			
potF	putrescine-binding periplasmic protein	stress, multiple	periplasmic space, membrane			
potD	polyamine transp. Subunit/ binding compon. of ABC superfamily	stress, multiple	perplasmic space			
ydiY	predicted outer membrane protein, acid-inducible	stress, acid	outer membrane			
yagU	inner membrane protein yagU	stress, acid resist	inner membrane			
cutC	copper homeostasis protein CutC	stress, detoxific.	cytosol			
yjbJ	UPF0337 protein yjbJ	stress, osmotic	cytosol			
nrdH	hydrogen donor for NrdEF electron transport system	stress, oxidative	cytosol			
nrdI	flavodoxin required for NrdEF cluster assembly	stress, oxidative	cytosol			
osmC	lipoyl-dependent Cys-based peroxidase, hydroperoxide resist.	stress, oxidative	cytosol			
ecnB	entericidin B membrane lipoprotein	stress, cell death	inner membrane			
cysD	sulfate adenylyltransferase subunit 2	sulfate assimilation	cytosol			
cysH	phosphoadenosine phosphosulfate reductase	sulfate assimilation	cytosol			
cysJ	sulfite reductase [NADPH] flavoprotein alpha-component	sulfate assimilation	cytosol			
cysA	sulfate/thiosulfate import ATP-binding protein CysA	sulfate assimilation	cytosol, inner membr.			
cysU	sulfate/thiosulfate transporter subunit	sulfate assimilation	inner membrane			
cysP	thiosulfate-binding protein	sulfate assimilation	perplasmic space			
codA	cytosine deaminase	nucleotide synth.	cytosol			
yjjZ	uncharacterized protein involved in nitrogen metabolism	uncharact. protein	cytosol			
ybgE	hypothetical protein ybgE	uncharact. protein	inner membrane			
ydjN	uncharacterized symporter ydjN	uncharact. protein	inner membrane			
yjiH	hypothetical protein yjiH	uncharact. protein	inner membrane			
ylaC	inner membrane protein ylaC	uncharact. protein	inner membrane			

Exponential growth

14 genes were allocated to COG category J (translation) and two more genes (*rpmC* and *rpmI*) could be identified to encode components of ribosomal subunits as well, meaning that they have to be assigned to the same functional category. The common positive deregulation of genes (***rpl***, ***rpm*** and ***rps***) encoding components of ribosomal subunits indicates a markedly increased translational activity. It, therefore, has to be assumed that cultivation in milk whey rather than in DMEM resulted in increased growth of both mastitic isolates. One explanation might be that DMEM initially provided good nutritional conditions while *E. coli* that were introduced into milk whey needed more time to adapt to the altered nutrient composition and availability. Consequently, this might have caused an extended lag-phase relative to that of *E. coli* grown in DMEM. Another possibility might be that *E. coli* mastitis isolates sense the presence of particular components of milk whey and react in a way trying to outrun the antimicrobial conditions by increased growth rates.

Carbon compound and energy metabolism

Increased growth rates are a common and advantageous attribute of ExPEC when new niches have to be colonized. It has been suggested that adaptation to new main carbon sources also alters the transcriptional profile of carbon metabolism-related genes. The ***fruK*** gene represents the only gene associated with carbon catabolism, which was negatively deregulated in milk whey or in milk whey +LPS relative to DMEM. Specifically, *fruK* encodes the 1-phosphofructokinase, which is not only one of the most important regulatory enzymes of glycolysis, but also essential for the utilization of fructose as a carbon source (Buschmeier et al., 1985). It should be noted that glucose and fructose phosphotransferase systems (PT-systems) are constitutively expressed in *E. coli*. The decreased expression of *fruK* suggests that both *E. coli* mastitis strains have indeed adapted to altered carbon sources during 1h of incubation in milk whey where glucose concentrations are substantially lower than in DMEM. Additionally, the phosphoglycerate mutase-encoding gene ***gpmA,*** which is also involved in glycolysis, was down-regulated in milk whey after LPS challenge. On the contrary, other genes involved in primary carbon energy metabolism, such as ***aceE***, ***frdD***, ***lipA*** and ***ptsG*** were positively deregulated mainly in milk whey +LPS. A possible explanation may be that these genes were required for the utilization of carbon sources that have become available or that were preferred in contrast to carbon sources that were diminished. Moreover, it can be speculated that *E. coli* which encountered increased adverse conditions, at least in comparison to growth in DMEM, are likely to be in need for more energy to compensate for the adaptation to these conditions and the increased transcription. The required energy might be provided by the pyruvate dehydrogenase AceE (supported by cofactor lipoate from LipA lipoatesynthase) linking the glycolysis to the citric acid cycle and, consequently, releasing energy via NADH. At least ***nuoA*** encoding for NADH:ubiquinone

oxidoreductase subunit A was positively deregulated in pure milk whey as well and further corroborates the impression of enhanced energy metabolic activity.

Citrate: a readily available and utilized carbon source in mammary secretions

Besides deregulated genes of the primary carbon energy metabolism, genes of the ***cit*** operon (*citM-**citCDEFXG***) have been positively deregulated in milk whey and in milk whey +LPS. Increased expression of the *cit* operon, which encodes the citrate lyase complex, implies that its transcription enabled utilization of citrate as a carbon source. In the past, different coliform bacteria that cause mastitis were characterized by their mobility and their ability to utilize citrate. Accordingly, *E. coli* was supposed to be a citrate-negative microorganism which cannot grow on citrate as the sole carbon source (Hogan and Smith, 2003; Hogan et al., 1999). However, citrate-positive *E. coli* isolates from the environment, humans, feral birds, domestic animals and cattle have been reported decades before (Ishiguro et al., 1979; Ishiguro et al., 1978). In *E. coli*, the *cit* cluster comprises the genes encoding the citrate lyase (*citDEF*), which we found to be up-regulated and *citT* coding for the citrate/succinate antiporter CitT (**Figure 35**). In contrast to *E. coli*, other bacteria are able to grow on citrate as the sole carbon and energy source (Martín et al., 2004). Because *E. coli* lacks the genes encoding the oxaloacetate decarboxylase, its citrate fermentation is dependent on an oxidizable co-substrate (Lütgens and Gottschalk, 1980). Lütgens and Gottschalk found that citrate was fermented by citrate-positive *E. coli* if a second substrate, such as glucose, lactose or lactate, was present. Knowing that citrate and lactose are abundant in mammary secretions, the deregulation of the *cit* cluster in milk whey and milk whey +LPS reflects a metabolic adaptation to citrate as a substantial carbon source in mammary secretions. It is further suggested that the presence of the *cit* cluster enables *E. coli* to multiply independently from carbon and energy sources other than citrate and lactose, thus providing a growth advantage over *E. coli* that lack the *cit* cluster.

It has, nonetheless, to be admitted that neither *citA* nor *citB* were deregulated in milk whey or milk whey +LPS compared with the DMEM sample, although the *cit* cluster is known to be regulated by the sensor kinase CitA and the response regulator CitB (Bott, 1997). Thus, it is recommended to investigate various *E. coli* isolates, from bovine mastitis and bovine faecal samples, particularly with regard to a correlation of the presence and deregulation of the *cit* cluster with an increased fitness of *E. coli* mastitis isolates.

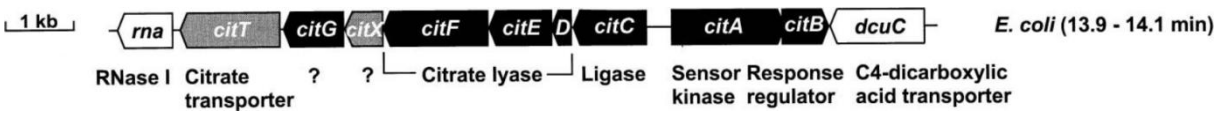

Figure 35: *E. coli* genes required for citrate fermentation. Genes shaded in light gray are those present only in the *E. coli cit* cluster, but not in *cit* clusters of *K. pneumoniae*, *L. mesenteroides*, *W. paramesenteroides* and *L. lactis*. The *citDEF* genes, coding for the citrate lyase, are highly conserved in the different *cit* clusters. Modified scheme as published by (Pos et al., 1998).

Amino acid biosynthesis

As shown in **Figure 34**, the majority of the genes with significant changes after incubation in milk whey and in milk whey +LPS were required for amino acid biosynthesis or -utilization. In contrast to the 19 deregulated genes, that have been associated with amino acid biosynthesis, transport and metabolism according to the COG classification, four genes of that category were reassigned to other processes, because of their biological functions: *cysD* and *cysH* were allocated to sulfate assimilation, and *potD* and *potF* were grouped to stress related determinants. The 15 remaining genes were individually assigned to different amino acid pathways

Arginine biosynthesis: The deregulated genes ***argB***, ***argC***, ***argI***, ***argJ***, ***argT***, ***artI*** and ***artJ*** reflect arginine biosynthesis. Most genes (*argC*, *argI*, *argJ*, *argT* and *artJ*) were up-regulated, whereas *argB* was exclusively down-regulated and *artI* was exclusively up-regulated in milk whey +LPS relative to DMEM. Both genes were not deregulated in milk whey compared to DMEM. As mentioned before, *artJ* expression was approximately two-fold increased in milk whey compared to its up-regulation in milk whey +LPS (corroborated by its down-regulation in milk whey +LPS relative to milk whey). Thus, arginine biosynthesis has clearly been influenced to a greater extend by incubation in milk whey while the same influence was apparently abolished, at least altered, in milk whey +LPS. We propose that arginine biosynthesis is considered subordinate when mastitic *E. coli* are forced to cope with the conditions in LPS-challenged mammary secretions, in which the concentration of antimicrobial components are increased. Nevertheless, genes involved in arginine biosynthesis were deregulated in both milk whey and milk whey +LPS, suggesting that the ability to synthesize or acquire arginine in milk whey is important. It raises the question what virulence- or fitness-associated function arginine biosynthesis may convey during the initial stages of *E. coli* mastitis? Regarding its role in human ExPEC, a previous study on UPEC reported a marked increase in *artJ* expression when grown in human urine. Because arginine concentrations are limited in urine, they emphasized that the ability of *E. coli* to synthesize or acquire arginine is important for virulence in the urinary tract (Russo et al., 1999). This was supported by the notion that experimentally determined arginine concentrations in adult urine ranged from 3.8 to 90 nmol/ml and resulted in reduced growth of *E. coli,* decreased by 50 to 90 % of the maximum population density (Russo et al., 1996). Nevertheless, arginine limitation is unlikely to occur in mammary secretions. Although the *de facto* arginine concentration of the milk whey used in the present study has not been evaluated, the approximate arginine concentration in nonfat milk is about 7.3 µmol / ml according to http://nutritiondata.self.com. Thus, it can be assumed that it is 245-fold higher in milk whey than in human urine. Here, a better explanation for increased arginine biosynthesis would probably be that arginine is prerequisite for one of the three main enzyme-based acid resistance pathways comprising the arginine-decarboxylase pathway (Zhao and Houry, 2010). The increased transcription of biosynthetic enzymes providing arginine for further

utilization in the acid stress response would also be in line with the positively deregulated genes *ydiY* and *yagU* in milk whey.

Glutamine / glutamate biosynthesis:

Besides the arginine-decarboxylase pathway, the glutamine- and lysine-decarboxylase pathways are utilized in acid resistance. We found a 3 to 4-fold increase of the transcriptional level of deregulated genes involved in glutamine biosynthesis (***glnH***, ***glnP***, ***glnQ***, ***gltI*** and ***gdhA***) in milk whey and in milk whey +LPS relative to DMEM. A marked difference between the corresponding expression levels in milk whey and milk whey +LPS was not detected. Utilization of glutamine in enzyme-based acid resistance pathways is in line with other observations described before. Moreover, *E. coli* temporarily accumulates glutamate, which can be obtained from glutamine, as a compatible solute to outweigh the K^+-ions influx due to hyperosmotic conditions (Koegel, 2008). This is further in line with the positively deregulated expression of *gdhA*, which is involved in glutamate biosynthesis. Expression of *gdhA* is increased upon hyperosmotic stress when glutamate dehydrogenase encoded by *gdhA* can provide a steady-state to external pH effects.

Increased **peptidase activity** is reflected by increased transcriptional level of the genes ***iaaA*** and ***pepE*** which both encode peptidases specific for N-terminal aspartic dipeptides. The *in vivo* contribution of the *iaaA* and *pepE* gene products to the utilization of N-terminal aspartic peptides is unknown, but allowed *Salmonella* strains not deficient of N-terminal aspartyl hydrolyzing peptidases to grow on aspartyl-leucine as a leucine source. Moreover, *iaaA* represents the first gene of an operon that also encodes a putative ATP-binding cassette transporter suggesting that peptide catabolism is an additional function of IaaA (Larsen et al., 2001). As the complex nutritional conditions in milk whey and in milk whey +LPS remain to be analyzed in detail, we might emphasize that the *iaaA* and *pepE* gene products contribute to the transport and utilization of aspartic peptides in *E. coli* to enable the utilization of additional sources of amino acids. The idea that *E. coli* 1303 and ECC-1470 have to rely on other amino acid sources in mammary secretions is supported by the marked negative deregulation of ***yhaO*** in milk whey and in milk whey +LPS. The *yhaO* gene encodes a still uncharacterized member of the STP transporter family, which is located downstream of the *tdc* operon including the gene for the TdcC threonine transporter of the STP family. It can be speculated, that the utilization of compounds transported by YhaO, is less beneficial in milk whey than it might be in DMEM.

Altogether we can state, that the deregulated genes that can be correlated with amino acid biosynthesis or processing reflect first processes associated to stress response, and secondly an adaptation to altered nutritional conditions. Whether the particular transcriptional profiles in milk whey and in milk whey +LPS have been influenced by either the inhibitory traits conveyed by milk

whey or whether they represent a more general adaptation to the growth medium in terms of nutrition and biochemical milieu, needs to be verified.

Sulfate assimilation

Several genes under control of the ***cys***teine regulon were found to be positively deregulated in milk whey compared to DMEM, but showed no deregulation in milk whey +LPS relative to DMEM. *cysAUWcysP* codes for a sulfate transporter that belongs to the ATP-Binding Cassette (ABC) superfamily of transporters (Wu and Mandrand-Berthelot, 1995). Its expression enables utilization of both sulfate and thiosulfate as a sole sulfur source, but does not correspond to the challenges provided by milk whey or milk whey +LPS discussed before. Moreover, the expression of the genes *cysJH* was in line with expression of the other deregulated *cys* genes, though they belong to a different transcriptional unit. Expression of *cysJH* is known to be inhibited by oleanolic and ursolic acid which in turn affect the expression of the cysteine regulon and the stress response (Grudniak et al., 2011). So far, a possible role in stress response is the only function that can be anticipated in the present context. The expression profile of the deregulated *cys* genes can, nonetheless, not be correlated to particular processes other than stress.

Virulence associated factors

Virulence factors could not be identified as to be encoded among the significantly deregulated genes, although two genes (*ygeO* and *tsx*) with a virulence-associated function appeared to be deregulated. The gene ***ygeO*** encodes a type three secretion system (**T3SS**) apparatus protein, but its gene product was also found to be involved in the generation of an *E. coli* extracellular death factor (**EDF**) (Kolodkin-Gal et al., 2007). Its negative deregulation in milk whey relative to DMEM leads to the suggestion that a conversion of EDF release occurred, specifically the MazF-MazE toxin-antitoxin system. It will have to be further elucidated what particular role the deregulation of *ygeO* expression plays in this context, especially because the MazF-MazE system causes programmed cell death in response to stresses including starvation and antibiotics (Aizenman et al., 1996; Sat et al., 2001).

The virulence-associated gene ***tsx*** was found to be positively deregulated in milk whey +LPS relative to DMEM, suggesting a specific bacterial response to compounds increased after LPS challenge. An early study reported that *tsx* encodes a nucleoside channel and functions as a receptor for bacteriophages and colicins (Hantke, 1976). The *tsx* gene product represents a porin protein and forms a channel that crosses cellular membrane. The structure, location and distribution on the bacterial surface convey different survival strategies to evade immune pressure and to respond to antibiotics, which is suggesting a significant role as a pathogenicity effector (Achouak et al., 2001). While down-regulation of *tsx* is not only related to, but also required for iron homeostasis in *E. coli* (Lin et al., 2008), consequences of *tsx* up-regulation are ill-defined. In addition to the negative

deregulation of genes associated with iron assimilation, the *tsx* down-regulation is in line with the finding that iron shortage appears to be no issue for *E. coli* mastitis strains in milk whey or milk whey +LPS.

Expression of bacterial regulators in milk whey and milk whey +LPS

Besides the expression of deregulated determinants, which are grouped together because of either biological function or localization in transcriptional units, specific transcriptional regulators were deregulated in response to milk whey and milk whey +LPS.

The ***cpxP*** gene product is a highly inducible, negative regulator of the *cpx* regulon. While extracytoplasmic stress is indicated by up-regulated expression of *cpxP* (Danese and Silhavy, 1998), its down-regulation in milk whey might indicate other hostile conditions to the bacterial cell envelope. Decreasing CpxP abundance caused has been shown important in Cpx pathway activation (Raivio et al., 2000). As CpxP overproduction turns off the *cpx* response by feedback inhibition (Raivio et al., 1999), a *cpx* response may be induced in turn when *cpxP* expression is down-regulated. It is possible that the Cpx system in *E. coli* is likewise involved in modulation of cell surface characteristics as has been suggested for UPEC strains. There, amongst other functions, the Cpx systems regulates P-fimbriae expression (Hernday et al., 2004; Hung et al., 2001; Jones et al., 1997). Therefore, deregulation of *cpxP,* as an inducible regulator of the Cpx response, seems to be involved in host-pathogen interaction although it remains hard to discern its particular contribution to ExPEC pathogenicity in general and mastitis in particular. Besides up-regulation of master regulator gene *flhC* required for motility and flagella biogenesis (Stafford et al., 2005; Claret and Hughes, 2002), we found no indications for deregulation of cell-surfaces appendages like flagella, pili and fimbria. It can be hypothesized that the down-regulation of cpxP in milk whey might be involved in resistance to extracytoplasmic stresses (Danese and Silhavy, 1998). Whether the Cpx system regulates expression of virulence traits or maintains and modulates the bacterial cell surface in the presence of antimicrobial compounds in mammary secretions needs to be elucidated. The Cpx system is at least part of a complex network of signaling cascades linked to virulence, various biosynthetic and metabolic pathways (Debnath et al., 2013; Hunke et al., 2012; Jung et al., 2012; Buelow and Raivio, 2010; Gerken et al., 2010; Price and Raivio, 2009; Wolfe et al., 2008; Dorel et al., 2006; Raivio et al., 2000; Raivio et al., 1999).

The ***flhC*** gene was positively deregulated in milk whey +LPS, but not in milk whey relative to DMEM. The FlhC protein is part of the transcriptional dual regulator FlhC-FlhD, which constitutes the master regulator of gene expression required for motility and flagella biogenesis (Stafford et al., 2005; Claret and Hughes, 2002). Although both traits were discussed to be advantageous for *E. coli* when passing the teat canal as a physical barrier, the genes belonging to the flagellar operons have not been detected to be deregulated in *E. coli* mastitis isolates 1303 and ECC-1470. We might speculate that

increased expression of *flhC* is affecting cell division, biofilm formation and virulence as reported for EHEC. In earlier physiological experiments it was confirmed that FlhC reduced the cell division rate, the amount of biofilm biomass and pathogenicity in a chicken embryo lethality model (Sule et al., 2011). The deregulation of *flhC* as part of the *flhDC* master operon can, however, be induced in response to various stimuli including synthesis of type 1 fimbriae, quorum sensing, high osmolarity and catabolic repression (Lehnen et al., 2002; Sperandio et al., 2002; Shin and Park, 1995; Soutourina et al., 1999). Our transcriptome analysis could neither confirm nor exclude any of these possibilities.

Besides *flhC*, the only positively deregulated regulator-encoding gene was ***hycA***, which was exclusively deregulated in milk whey, but not in milk whey +LPS. The *hycA* gene product is known to be the regulator of the transcriptional regulator *FhlA*. It participates in controlling several genes involved in production of the formate hydrogenlyase system. The specific regulative mechanism by *HycA* is unknown. Expression of *hycA* is activated by formate. HycA may directly interact with the FhlA protein and/or prevent the binding of FhlA to activator sequences (Suppmann and Sawers, 1994; Sauter et al., 1992; Leonhartsberger et al., 2000; Skibinski et al., 2002). Consequently, we emphasize that *hycA* expression of *E. coli* in mammary secretions might be involved in anaerobic respiration. Its regulation target FhlA is required for regulation of expression of the *hydN-hypF* operon, which has been discussed on page 101.

The DNA-binding transcriptional repressor gene ***iscR*** was down-regulated not only in milk whey +LPS relative to milk whey, but also in milk whey +LPS relative to DMEM. In particular the "**I**ron **s**ulfur **c**luster **R**egulator" is negatively autoregulated and comprises an iron-sulfur cluster that acts as a sensor of iron-sulfur cluster assembly (Fleischhacker et al., 2012). In this way, IscR regulates expression of more than 40 genes and further expression of operons responsible for a secondary pathway of iron-sulfur cluster assembly, iron-sulfur proteins, anaerobic respiration enzymes and also biofilm formation (Tokumoto and Takahashi, 2001; Schwartz et al., 2001; Giel et al., 2006; Lee et al., 2008; Yeo et al., 2006; Wu and Outten, 2009). It has been reported that *iscR* expression is up-regulated during biofilm growth of UPEC in urine compared to that during planktonic growth in MOPS and urine (Hancock and Klemm, 2007). Considering these functions, it is hard to discern which role IscR particularly plays in milk whey +LPS.

The ***rmf*** gene has been negatively deregulated in milk whey samples. This gene does not encode a transcriptional regulator, but a "**R**ibosome **m**odulation **f**actor". Rmf reversibly converts active 70S ribosomes into dimers during the transition from exponential growth to stationary phase. This conversion is associated with a decrease in overall translation activity (Wada et al., 1990 and 1995). In this analysis, up-regulation of ribosomal subunit genes (*rpl*, *rpm* and *rps*) indicated exponential growth in milk whey samples relative to DMEM. Therefore, the decreased transcription rates of *rmf* in milk whey and milk whey +LPs relative to DMEM are in line with this observation.

We successfully identified regulator gene ***codA*** to be increased in expression in milk whey +LPS in comparison to the DMEM samples although no deregulation was determined in milk whey. The *codA* gene is encoding for a cytosine deaminase, which has an enzymatic function in nucleotide and nucleoside conversions of the pyrimidine salvage pathway (Ahmad and Pritchard, 1972; de Haan et al., 1972). It is known that *codA* expression is increased by growth on poor nitrogen availability (Andersen et al., 1989). The regulation of the *codBA* operon was more recently described to be dependent on the nitrogen assimilation control protein NAC (Muse et al., 2003). However, deregulation of the *nac* gene was not determined in any of the samples investigated in this study. No deregulation of *nac* suggests another reason for *codA* deregulation, but on the other hand it is ill-defined whether *nac* deregulation is required for regulation of *codBA* expression. Apart from that, the *codBA* operon seems to be regulated as well by the UTP level of the bacterial cell (Turnbough and Switzer, 2008). Accordingly, it is difficult to determine exactly which condition led to up-regulation of *codA* expression. Nevertheless, *codA* up-regulation in the context of LPS-challenged milk whey represents an interesting approach to interfere with *E. coli* growth in udder tissue. In detail, the *codA* gene product is present in prokaryotes, but not in eukaryotes and, thus, CodA may represent a target for antimicrobial agents. So far, the cytosine deaminase is being used for suicide gene therapy against tumors (Mullen et al., 1992; Austin and Huber, 1993; Dong et al., 1996; Springer and Niculescu-Duvaz, 1996). While antibodies against cytosine deaminase are commercially available and *codA* is currently utilized as a negative selection marker for gene disruption in *Streptomyces* and other bacteria (Dubeau et al., 2009), the use of CodA for controlling *E. coli* mastitis should be considered.

Stress-related bacterial determinants

It is postulated that *E. coli* colonizing the mammary gland, will take advantage of fitness traits, which they already possess. It has also been discussed that once present in mammary secretions, *E. coli* is challenged by host factors and altered nutritional conditions that cause stress. Several examples of stress response have been observed in our study based on differential expression of particular determinants or regulators such as Cpx were discussed in this chapter. Additionally, we successfully identified stress-specific determinants that were deregulated as well.

Acid stress

Only two deregulated genes directly associated with stress were up-regulated. In milk whey +LPS it was ***ydiY***, which has not been characterized so far. The sequence similarity of hypothetical protein YdiY suggests that it is a member of the outer membrane receptor (OMR) family (Zhai and Saier, 2002). Various virulence-associated determinants appear to be membrane-associated or even exposed on the bacterial cell surface. Due to the fact that *ydiY* was reported as one of two genes,

which were induced when *E. coli* was grown at acidic pH (Stancik et al., 2002), it is likely involved in bacterial adaptation by sensing environmental conditions. While acid stress response dependent on the *ompC* and *ompF* gene products is well described (Pratt et al., 1996; Heyde et al., 2000; Sato et al., 2000), there is no further information about the biological role of YdiY by today. This and its up-regulation in response to milk whey +LPS, makes it an interesting candidate that possibly confers increased fitness to mastitis and other pathogenic *E. coli* isolates. We have to admit, however, that no deregulation of *ompC* and *ompF* could be determined in either *E. coli* 1303 or ECC-1470 when incubated in milk whey or milk whey +LPS, although the presence of these genes in both genomes has been confirmed. This is not necessarily a contradiction, because the bacterial stress response results from a complex regulatory network allowing the bacteria to fine tune their response to the external environment.

Another gene that could be associated to acid stress and that has been positively deregulated in pure milk whey is ***yagU***. Like for *ydiY*, there is no information about *yagU* other than that its encoded protein comprises a transmembrane region and the gene itself is induced by acidic pH (Kannan et al., 2008; Hayes et al., 2006). As both *ydiA* and *yagU* appear to be up-regulated, in either milk whey or milk whey +LPS, it might be interesting to investigate whether the encoded membrane-associated proteins confer sensitivity or even resistance to hostile conditions within the mammary gland. On the other hand, it has to be admitted that mastitis *E. coli*, unlike many IPEC, do not have to encounter gastric acid or bile salts.

In contrast to *ydiA* and *yagU*, the majority of deregulated genes that could be directly related to stress exhibited consequent negative deregulation. Unfortunately we cannot offer an explanation for the negative deregulation of ***cutC*** in milk whey +LPS. This gene codes for a copper homeostasis protein, but there is no information available that connects to detoxification traits advantageous in coliform mastitis.

Osmotic stress

Another gene that was negatively deregulated in milk whey +LPS is ***yjbJ***. Under control of the σ^S regulon, its expression is induced under hyperosmotic stress imposed by NaCl under aerobic and anaerobic conditions (Weber et al., 2005 and 2006). This seems to be contradictory to the consequent up-regulation that was observed for genes of the arginine- and glutamine-decarboxylase pathways when incubated in milk whey and milk whey +LPS. Additionally, we did not determine any deregulation of *rpoS* encoding σ^S. Again, we refer to the fact that *E. coli* stress response does not rely on single determinants, but includes a complex network, which reacts to different stresses. The ***yjbJ*** gene product has been identified as an appropriate biomarker for matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry (TOF-MS) and, thus, contributes to protein-taxonomic classification of pathogenic bacteria (Fagerquist et al., 2010).

Another osmotic stress-associated gene that we found to be negatively deregulated in milk whey and milk whey +LPS, is ***ecnB***, which is part of the entericidin locus of *E. coli*. While the entericidin A lipoprotein EcnA represents the antidote, EcnB is the bacteriolytic entericidin B lipoprotein of EcnAB, a linked toxin-antitoxin "addiction module" (Bishop et al., 1998). Together, EcnAB enables regulation of bacterial programmed cell death under high osmolarity conditions. The marked down-regulation of *ecnB* in milk whey, relative to DMEM, is once more suggesting that no osmotic stress is about to elicit a corresponding response, probably due to the physiologic composition of milk whey itself.

Oxidative stress

It can be anticipated that bacteria approaching mammary secretions are exposed to reactive oxygen species (ROS), produced by phagocytes, which directly and indirectly support killing of the approaching bacteria (Thannickal and Fanburg, 2000). Moreover, ferrous iron is oxidized by ROS and, thus, causes inactivation of iron-cofactored key enzymes in metabolic redox-reactions (Jang and Imlay, 2007; Park et al., 2005). Specifically, the *E. coli nrdHIEF* operon encodes for the alternative ribonucleotide reductase NrdEF. The manganese-dependent enzyme NrdEF allows for cell replication during iron starvation caused by ROS when the iron-dependent ribonucleotide reductase NrdAB is functionally diminished (Martin and Imlay, 2011). We found that *nrdH* and *nrdI*, both members of the *nrdHIEF* operon, have been negatively deregulated in milk whey and milk whey +LPS. Specifically, *nrdH* encodes a thiol-based redoxin, which replaces the thioredoxin in ribonucleotide reduction, while *nrdI* encodes the flavodoxin NrdI involved in cofactor maintenance of the ribonucleotide reductase (Jordan et al., 1997; Cotruvo and Stubbe, 2008). As the *nrdHIEF* operon is highly expressed during oxidative stress (Monje-Casas et al., 2001; Martin and Imlay, 2011), down-regulation of both these genes suggests that no oxidative stress existed in our samples, at least after 1h incubation in mammary secretions. This is further corroborated by the negative deregulation of ***mntH*** in milk whey and milk whey +LPS. Its gene product, the manganese importer MntH, is required by the manganese-dependent NrdEF. During oxidative stress conditions *mntH* is strongly induced (Martin and Imlay, 2011; Kehres et al., 2002). At least, the isogenic mutants of regulatory gene *iscR*, which we as well determined to be down-regulated in milk whey +LPS, have been shown to elicit a decrease in *nrdHIEF* operon transcription (Martin and Imlay, 2011).It has been further suggested that the regulators Fur and IscR are opposed regulators for *nrdHIEF* when oxidative stress mutilates the bacteria. In this case, IscR might activate while Fur represses transcription of *nrdHIEF.* Furthermore, the negative deregulation of *osmC* is in line with the finding of no oxidative stress conditions at 1h in milk whey +LPS. The *osmC* gene product confers peroxidase activity and has been determined sensitive to oxidative stress (Lesniak et al., 2003; Conter et al., 2001). We suggest that at least 1h hour after the beginning of the incubation in milk whey and milk whey +LPS, the transcriptional response of *E. coli* mastitis strains 1303 or ECC-1470 does not indicate any oxidative stress. Albeit the presence of ROS

in milk whey can be anticipated, their concentration may be below the harming threshold. This may be due to the fact that milk whey does not comprise any macrophages for ROS production. In this case, the negative deregulation of genes associated to oxidative stress can be seen as a feedback signal. Thus, it can be concluded that oxidative stress has been overcome by both mastitis strains at the very onset of incubation in milk whey.

Multiple stresses

The genes *potD* and *potF* belong to the *potFGHI* operon of the putrescine transport system in *E. coli* (Pistocchi et al., 1993). These genes have been positively deregulated in milk whey and milk whey +LPS. Due to the ill-defined relation of putrescine transport, a particular role in putrescine catabolism or anabolism is difficult to anticipate. From what is known, putrescine facilitates mRNA translation and, thus, contributes to cell viability controlled by Rmf (Terui et al., 2010). Anyhow, the Rmf-encoding gene was down-regulated as well in our *E. coli* transcriptome samples from milk whey and milk whey +LPS, the putrescine transport system has also been referred to as a bacterial periplasmic transport system. While ribosome modulation occurs in the cytosol, it can be emphasized that transcription of the putrescine transport system is more related to another function conferred by putrescine. It has been discussed previously, that in addition to amino acid synthesis, the release of putrescine is indicating hyperosmotic stress (Koegel, 2008). Furthermore, putrescine is a polyamine, which can confer various functions apart from effecting bacterial growth. For example, polyamines seem to be involved in the regulation of biofilm development in bacteria (Wortham et al., 2007). It is common knowledge that biofilm formation is a protective encasement method to shield the bacteria against harsh environmental conditions. This way or another, the identified expression of the putrescine transport genes might reflect stress-associated processes in both mastitis *E. coli* strains. A recent study supports that putrescine catabolism is a metabolic response to several stresses in *E. coli*. Among others, nitrogen-limited growth often involves utilization of nitrogen sources other than an ammonium salt, such as putrescine or arginine (Schneider et al., 2013). At least the particular role of polyamines during anaerobic growth is still unknown.

V.6.7. Remarks on RNA-Seq

In the present study we have used high-throughput Illumina sequencing of cDNA, referred to as RNA-Seq to comprehensively analyze the transcriptomes of two *E. coli* isolates from bovine mastitis. Previous studies have already proven that RNA-Seq expression measures are highly reproducible by qRT-PCR, DNA-microarray and protein expression data obtained by shotgun mass-spectrometry (Nookaew et al., 2012; Marioni et al., 2008; Fu et al., 2009). The transcriptome data of the present study provided sufficient mRNA expression levels for the successful identification of DEG in mastitic *E. coli* incubated in milk whey to mimic conditions during mastitis infection.

It should, nevertheless, be noted that serious difficulties might appear when combining different sequencing technologies in RNA-Seq-based identification of differentially expressed genes: The identification of differentially expressed genes requires high quality genome data and RNA-Seq data, since it is well known that accurate mapping is mandatory to estimate gene expression levels (Nookaew et al., 2012). From our investigation we can conclude that sequence reads obtained by the Illumina platform can hardly be mapped to reference sequences acquired by 454 sequencing technology, because the 454 reference sequences are commonly corrupted by incorrect homopolymer runs. In consequence, the mapping parameters have to be widened and this subsequently affects correct read mapping and inflicts bias to the estimation of expression levels. Finally, not only the identification of differentially expressed genes, but the whole transcriptome analysis might be biased as well.

Bottom line, RNA-Seq based transcriptomic is a great advantage in understanding the molecular basis of infection and disease caused by *E. coli*. Furthermore, we hope that the acquired transcriptome datasets will be a useful resource to broaden the knowledge of the *E. coli* research community.

VI. GENERAL DISCUSSION

The present study aimed at the characterization of virulence-associated traits from *E. coli* model mastitis isolates 1303 (acute mastitis) and ECC-1470 (persistent mastitis). It is assumed that virulence-associated traits of *E. coli* mastitis isolates are deregulated during *E. coli* growth and colonization of the bovine udder. Multiple parameters can affect the transcriptional profiles of *E. coli* during adaption. *E. coli* invading the bovine udder encounter drastically altered environmental conditions as well as several host defense mechanisms. Besides the cellular components of the immune response, the invading bacteria are challenged by soluble components of the immune system. These components mainly comprise numerous defensins, lysozyme, lactoferrin and lactoglobulin (Kawai et al., 2013; Piccinini et al., 1999; Chaneton et al., 2008). Moreover, nutritional limitations (e.g. iron, nitrogen, carbohydrates etc.) and altered environmental cues, such as oxidative agents, osmotic and pH changes, might even challenge the highly versatile *E. coli*. By facing these harsh conditions, *E. coli* has to successfully sense and quickly adapt. Therefore, *E. coli* needs to respond by appropriate regulation of fitness and virulence-associated traits.

In order to identify relevant phenotypes or subgroups among the *E. coli* isolates from mastitis cases and from bovine faeces, phenotypic assays have been employed. Additionally, the effect of selected milk components was tested. We generated reproducible data by phenotypic assays and transcriptome analyses from bacteria incubated in milk whey. Last but not least, the bacterial transcriptome in the presence of bovine mammary gland epithelial cells was evaluated.

VI.1.1. *E. coli* 1303 and ECC-1470 lack particular virulence-associated genes

By focusing on two well-known *E. coli* mastitis strains, *E. coli* strain 1303 (acute mastitis isolate) and ECC-1470 (persistent mastitis isolate), we screened for the presence of virulence factors previously associated with mastitis (**Table 9**). It was found that *E. coli* 1303 and ECC-1470 differ in their individual virulence gene content. Specifically, *E. coli* 1303 and ECC-1470 lack particular virulence genes, which have been correlated with mastitis isolates in previous studies. On the one hand, the literature consequently reports rather low frequencies of virulence-associated factors that often do not exceed 30 % prevalence (Blum and Leitner, 2013; Silva et al., 2013; Cheng et al., 2012; Dogan et al., 2012; Kerro Dego et al., 2012; Fernandes et al., 2011; Suojala et al., 2011; Ghanbarpour and Oswald, 2010; Dyer et al., 2007; Wenz et al., 2006; Lehtolainen et al., 2003; Kaipainen et al., 2002; Wise et al., 2002; Lipman et al., 1995; Sanchez-Carlo et al., 1984). On the other hand, the total virulence potential of a pathogen is often greater than the sum of the individual contributions of virulence associated traits. Considering the fact that pathotypes have been defined by the site of infection / the niche in which they cause disease, it is widely accepted that virulence and fitness traits act synergistically and enable the pathogen to thrive. It can, therefore, be anticipated that the

bacterial determinants found to be deregulated in this study belong to a much bigger set of factors that promote a successful colonization of the udder. Regarding our transcriptome data from incubation in milk whey, the lack of positively deregulated and also of true virulence-associated determinants in both of the mastitis isolates indicated that *E. coli* might have adapted by other means to the udder (or at least mammary secretion) as an inflammatory site. We added interesting aspects and further knowledge to this topic.

VI.1.2. Environmental isolates provide a reservoir of potential mammary pathogenic *E. coli*

A previous study reported about a subset of environmental isolates, isolated from cows' environment in dairy farms, resembling mastitis isolates according to their growth, but the authors used pasteurized milk so that antimicrobial factors were probably neutralized (Blum et al., 2008). Thus, a particular causality remains unclear. In the present study, it has been focused on the phenotypic characterization of mastitis *E. coli* isolates relative to isolates from bovine faeces. We specifically addressed the question whether mastitis and faecal strains differ in their individual fitness traits under selected conditions mimicking those in mammary secretions. Antimicrobial factors that *E. coli* has to encounter during colonization of the udder originate either from blood serum or are synthesized in the mammary gland (Sordillo and Streicher, 2002). It is known that all of them are heat labile to different degrees and will be inactivated during milk processing, which includes severe heat treatment and drying, so that antibacterial factors are probably neutralized or confer decreased efficiency (Reiter and Brock, 1975).

In the present study, native milk whey was prepared from raw whole milk to assess the different phenotypic properties and transcriptional changes of selected *E. coli* isolates upon growth in mammary secretions. It has clearly been demonstrated that milk whey exhibits antimicrobial effects that are not provided by milk fat compounds or components of the cellular immune response. These antimicrobial effects are thought to result from either defensins, lysozyme, lactoferrin and lactoglobulin (Kawai et al., 2013; Isobe, Nakamura, et al., 2009; Piccinini et al., 1999; Chaneton et al., 2008), whereas *E. coli* revealed to be unsusceptible to lactoglobulin (Chaneton et al., 2011). We also challenged selected isolates with iron shortage, lactose as sole carbon source and LAP as a main defensin of milk, respectively.

Different outcomes of selected E. coli mastitis and faecal isolates in various phenotypic assays

It has been shown that in terms of fitness in milk whey, *E. coli* isolates from bovine faeces represent a heterogeneous group of which some isolates showed a substantially reduced ability to survive in milk whey (group 1), whereas others (group 2) phenotypically resemble mastitis isolates. In contrast, the *E. coli* mastitis isolates represented a phenotypically homogeneous group in that they showed similar survival and growth characteristics in milk whey. Interestingly, mastitis isolates did not exhibit such a

uniform phenotype in different other phenotypic assays conducted suggesting that these traits other than resistance to antimicrobial factors in milk whey are not critical for the establishment of mastitis, but they contribute to the overall fitness in milk whey. We clearly demonstrated that faecal isolates did not reveal any characterisitic, distinctive features which distinguish them from mastitis isolates except the reduced ability of group 1 isolates to survive in milk whey. Neither growth on lactose as the sole carbon source, the lactoferrin growth inhibition assay, nor the LAP resistance assay, was able to clearly distinguish group 2 isolates from mastitis isolates.

Following the individual growth characteristics of selected *E. coli* isolates in milk whey, it became obvious that initial survival is more important than growth rates. This can be concluded from the fact that the growth curves of mastitis and bovine faecal isolates turned out to resemble each other while the initial survival rates considerably differed. Milk whey includes a combination of antimicrobial components which may act synergistically or have additive effects. While of the determinants tested, LAP as a defensin contributed markedly to decreased bacterial counts, but had only weak effect on particular group 2 isolates, it has to be assumed that LAP and other defensins might act in synergy. It should be noted that resistance to defensins might be not a general molecular mechanism, but can be highly specific to the particular defensin. Besides LAP, there are several other defensins present in mammary secretions such as bovine neutrophil β-defensin, tracheal antimicrobial peptide, enteric β-defensin and bovine β-defensin, while the number of discovered defensins is still increasing (Kawai et al., 2013). Their synergistic action in contributing to the total antimicrobial effect of mammary secretions can be expected.

So far, we clearly demonstrated that bovine faecal *E. coli* isolates represent a heterogeneous group of which some isolates showed a substantially reduced ability to survive in mammary secretions (group 1), whereas others (group 2) resembled mastitis isolates that constitute a homogeneous group regarding their successful survival and growth characteristics in raw milk whey. Our results further corroborate the suggestion that some environmental isolates may provide a subgroup of *E. coli* with the potential to thrive in the bovine udder (Blum et al., 2008). Furthermore, we can summarize that encountering mammary secretions is a critical step of *E. coli* intramammary colonization. Ongoing bacterial growth and multiplication might be inhibited by components which are induced due to host response. Vice versa, bacterial fitness determinants have to be regulated during survival in milk whey.

VI.1.3. Encountering mammary secretions is a critical step to *E. coli* intramammary colonization

The primary colonization of the host or the inflammatory site in particular is regarded to be a critical step of an infection. Before mastitis causing *E. coli* are able to colonize the milk ducts and mammary

tissue, it is first of all important to survive and multiply in mammary secretions, which are known to contain antimicrobial compounds.

In the present study, we clearly demonstrated that milk whey provides antimicrobial effects independently from that of milk fat compounds or components of the cellular immune response. The bacterial transcriptional response to incubation in milk whey and milk whey +LPS has been assessed by RNA-Seq. Both, the total number and the number of exclusively deregulated genes in milk whey +LPS were considerably higher than those determined in milk whey in relation to the reference sample. Due to increased concentration of antimicrobial factors, because of the LPS challenge, this was expected and demonstrated that regulation of bacterial gene expression occurred in response to these antimicrobial factors. The identified differentially expressed genes of *E. coli* strains 1303 and ECC-1470 reflected distinct cellular processes including regulatory processes, amino acid biosynthesis and utilization, exponential growth, carbon energy metabolism, decreased iron utilization, sulfate assimilation, virulence and extracytoplasmic stress.

Stress response plays a vital role for initial survival

Encountering milk whey, the bacteria are challenged by multiple factors that simultaneously exert selective pressure. Consequently, *E. coli* isolates have to induce stress responses. The findings of the present study demonstrate regulation on various bacterial stress response genes including the Cpx stress response system. The observed deregulation of the expression of the glutamate and arginine pathways further corroborates the induction of stress responses upon incubation in milk whey. These pathways represent the main enzyme-based acid resistance pathways of ExPEC to compensate for acidic pH (Zhao and Houry, 2010).The release of glutamate and putrescine as solutes compensate for hyperosmotic stress (Koegel, 2008). The up-regulation of the putrescine transporter gene *potF* might support this suggestion. In line with this, acidic pH can be usually observed during exponential (and competitive) growth. We observed acidic pH as well in the presence of bovine mammary gland epithelial cells. Furthermore, we identified deregulation of *ydiY* expression that was reported as one of two genes induced when *E. coli* grows at acidic pH (Stancik et al., 2002). YdiY is likely to be involved in survival by sensing environmental conditions. Sensing environmental conditions is a prerequisite for pathogenic bacteria, as many of the newly encountered environments or niches confer potentially lethal mechanisms and the bacteria have to quickly sense and induce resistance against these lethal factors.

We anticipate that mastitis *E. coli* isolates modulate their cell surface characteristics by the Cpx system in order to improve resistance to extracytoplasmic stress. A similar situation has been observed in ExPEC in order to increase fitness and virulence (Debnath et al., 2013).

Interestingly, all deregulated genes identified, that account for oxidative stress response, were down-regulated at one hour after inoculation in milk whey and milk whey+LPS. We conclude, that oxidative

conditions caused by, e.g. ROS and/or NOS cannot be properly studied in milk whey, because ROS and/or iNOS are situationally expressed by cellular components of the host's immune response, which are depleted in milk whey. The obtained growth characteristics revealed similar curve shapes that suggest continued bacterial multiplication after the initial time period after inoculation in milk whey when defensins seem to be most active.

On the one hand, it is questionable whether these conclusions on antimicrobial effects in milk whey can be simply transferred to the *in vivo* situation. We emphasize that the causative agents that confer antimicrobial effects might constantly be released to different degrees in the mammary gland. The release of particular components such as defensins will even be increased (Isobe, Morimoto, et al., 2009). In contrast, in milk whey an initial concentration of these antimicrobial agents is provided which might be unleashed immediately. Consequently, the antimicrobial activity might be to some extent expended and provides a weak or even no further effect.

Bacterial entry into the udder will immediately elicit a bacterial response depending on the bacterial ability to sense the adverse conditions and regulate appropriate fitness traits. It is, therefore, also possible that quick adaptation and/or spontaneous, inheritable resistance to antimicrobial agents enable unimpeded growth although or even because these agents are further released. Interestingly, a previous study demonstrated that *E. coli* can become spontaneously resistant to AMPs upon *in vitro* exposure to slowly increasing peptide concentrations (Perron et al., 2006). It will be an interesting task to follow the bacterial resistance potential to different defensins. Thus far, we have not found any evidence for deregulation of genes encoding for extracellular proteases (such as DegP or EspP), which constitute obvious bacterial factors, which degrade AMPs. There are several other strategies to confer resistance to AMPs besides proteases. These mechanisms are often specific for certain peptides, relying on recognition and extracellular capturing or active extrusion of **C**ationic **A**ntimicrobial **P**eptides (CAMPs) from the bacterial membrane (Peschel and Sahl, 2006). Although neither resistance mechanisms nor their deregulation have been determined in the present study, it is likely that there is one or more that yet remain unexplored. To which extent such traits will be induced by the Cpx stress response system, as it has been reported for Tat-dependent peptidoglycan amidases conferring resistance to AMPs in *E. coli* and *Salmonella* (Weatherspoon-Griffin et al., 2011), remains to be investigated. Against this background, it remains a task to further discern the individual contribution of single factors in the milk whey to the total antimicrobial effect of milk whey. Together, multiple factors apply simultaneous selective pressure on *E. coli* initial survival and consequent growth. It has been successfully demonstrated for the first time that the initial thriving in milk whey represents a critical step in colonizing the bovine udder.

VI.1.4. Traits improving bacterial growth in mammary sectretion

Apart from antimicrobial agents, the limitation of essentials (i.e. by lactoferrin) represents an imminent inhibitory factor for bacterial multiplication.

Iron shortage does not occur to *E. coli* during first hours in mammary secretion

In our lactoferrin growth-inhibition assay, it has been shown that during 7 hours of incubation, lactoferrin provides no growth inhibiting effects to *E. coli* isolates either from mastitis (acute and persistent) or bovine faeces. Other studies reported on lactoferrin mediated bacteriostasis after markedly longer incubation (Rainard, 1986; Chaneton et al., 2011). Additionally, our transcriptional data on *E. coli* incubation in milk whey and milk whey+LPS indicate rather negative deregulation of genes associated with iron assimilation than an increased expression of iron uptake systems. Amongst others, genes of the enterobactin iron acquisition system were down-regulated. This is somehow surprising, because a previous study reported growth inhibition of *E. coli* in mammary secretions by blocking iron uptake with an antibody specific for the enterobactin receptor (Lin et al., 1999). This indicates the importance of iron uptake systems in the context of mastitis whereas it can be anticipated that other iron uptake or storage properties compensate for the reduced expression of enterobactin.

Furthermore, it has been hypothesized that bacteria approaching mammary secretions are exposed to ROS. ROS, produced by phagocytes, directly and indirectly support killing bacteria (Thannickal and Fanburg, 2000). Moreover, ferrous iron is oxidized by ROS and, thus, causes inactivation of metabolic key enzymes dependent on iron-redox-reactions (Jang and Imlay, 2007; Park et al., 2005). Consequently, alternative determinants are required to compensate for that. Vice versa, it can be concluded that the down-regulation of determinants that compensate for iron shortage indicates sufficient availability of iron. This might be a reasonable explanation for the fact that all differentially expressed genes associated with iron assimilation exhibited down-regulation in milk whey and in milk whey+LPS. This conclusion is supported by the fact that expression of the alternative ribonucleotide reductase NrdEF, which can compensate for the standard ribonucleotide reductase NrdAB during iron shortage, was down-regulated. In contrast to the iron-dependent NrdAB, the non-redox enzyme NrdEF uses imported manganese to replace iron (Martin and Imlay, 2011). A functional ribonucleotide reductase is, however, mandatory for aerobic growth (Jordan et al., 1996). The NrdEF supplementary manganese importer MntH can be considered as an indication for iron deficiency and it has been down-regulated as well in milk whey.

Altogether, we conclude that *E. coli* growth is not impaired by iron shortage in early stages of mastitis pathogenesis. Nevertheless, biologically accessible iron might be limited due to lactoferrin. Due to the fact that functional iron acquisition systems are considered to be essential in virulence, the observed negative deregulation of iron uptake associated genes is surprising. It has been shown

previously that iron availability altered the susceptibility of *E. coli* mastitis isolates to phagocytosis by neutrophils, but had no effect on their susceptibility to the bactericidal activity of blood serum (Wise et al., 2002). Whether a similar scenario can be assumed for mammary secretions remains yet unclear. Our observations suggest that either there is apparently no iron shortage to *E. coli* in milk whey or it confers less impact than initially thought. Especially, rather high concentrations of lactoferrin revealed no significant impact on vitality or growth of the isolates tested. These observations account for short term incubation in iron-limited media. Martin and colleagues reported that the manganese symporter protein MntH, required by NrdAB, failed to be expressed for several hours in iron-limited isogenic *nrdAB* mutants. They concluded that this might be the time needed for depletion of *E. coli´*s cellular iron stores (Martin and Imlay, 2011).

We have reasons to believe that iron may, however, become limited during later phases of *E. coli* growth in the mammary tissue. In parallel, a recent study reported that the inactivation of the biosynthetic pathways of several siderophores (enterobactin, salmochelin and yersiniabactin) abolished virulence of ExPEC in a mouse sepsis model indicating that these pathways are essential for the survival of ExPEC *in vivo* (Martin et al., 2013). Thus, we emphasize that expression of genes involved in iron assimilation might considerably differ between the initial and ongoing stages of mastitis pathogenesis. A first supplementary indication has been described by Lin and colleagues who reported that *E. coli* mastitis isolates share a specific enterochelin-iron retrieval system that includes the surface exposed protein FepA. It was expressed on all clinical mastitis isolates tested. A FepA-specific vaccine caused immune response in cows and blocked growth of *E. coli* in synthetic medium and dry mammary secretion (Lin et al., 1998 and 1999). Supporting our finding that iron is sufficient during initial bacterial growth in milk whey, we observed negative deregulation of the *fepA* gene in milk whey in relation to the DMEM reference sample. The only strictly iron-associated gene that was evidently up-regulated in milk whey +LPS, in contrast to the reference samples DMEM and milk whey, has been *ftnA*. The FtnA protein accounts for iron storage and might preserve iron, possibly in order to extent the time needed for depletion of *E. coli´*s cellular iron stores. We suggest that down-regulation of genes associated with iron uptake systems, when iron is still accessible or sufficient, might save energy costs to the pathogen. It can be anticipated that intramammary *E. coli* will quickly induce the down-regulated determinants that we determined, when iron becomes limited in ongoing stages of infection. A confirmation that rather late than early expression of iron uptake systems takes place is nonetheless required.

Together our results changed our view concerning the impact of iron on initial colonization during mastitis. It is also conceivable that this model of initial iron independency during transition from the environment to the host can be adapted to other ExPEC-caused diseases.

Citrate utilization might be a key factor to *E. coli* intramammary fitness

The milk ducts are a physiological environment with loads of antibacterial components and constant flow of mammary secretions which requires adhesion capability. From adhesion and invasion assays, it is suggested that a rather small portion of bacteria is internalized into the mammary tissue. In contrast, a major portion of the bacteria stays and multiplies in the milk ducts and caverns. Therefore, successful growth in mammary secretion requires adaptation to the nutritients available in milk. It is common knowledge that carbohydrates constitute a main energy source of *E. coli*. During our cocultivation experiments in the presence of host cells, the competition between the host cells and the bacteria for glucose and oxygen in the cell culture medium was a dominant feature. *In vivo* host cells are supplied by blood vessels while growing bacteria are initially depending on the carbohydrates present in mammary secretion. This suggests that an *in vivo* competition for nutrients is unlikely.

Citrate and lactose represent the main energy source available in mammary secretions in general. Due to the preparation of the milk whey from raw milk, both should have the same carbohydrate content. It has been discussed that the ability to utilize citrate might be important for mastitis *E. coli* isolates. Citrate and iron can be imported by the same way via the ferric di-citrate transport system (Fec) which we found to be deregulated upon growth in milk whey. By this, high citrate levels can compensate for the bactericidal effect of lactoferrin in milk due to the fact that citrate chelates iron and can then be taken up by the bacteria. Already imported, citrate becomes a valuable energy source, which itself depends on the ability to utilize iron. This trait is mediated by the *cit* operon. In the present study, a strong positive deregulation of the *cit* operon has been observed when mastitis *E. coli* isolates 1303 and ECC-1470 were incubated in either milk whey or milk whey +LPS. It should be noted that normally *E. coli* cannot grow on citrate as a sole carbon source under aerobic conditions and that this phenotypic trait, which has frequently been used to distinguish *E. coli* from other bacterial mastitis isolates (Quandt et al., 2014; Hogan and Smith, 2003; Hogan et al., 1999). However, citrate-positive isolates of *E. coli* from different environments, humans, feral birds, domestic animals and, moreover, cattle have been reported decades before (Ishiguro et al., 1979; Ishiguro et al., 1978). From the observed deregulation, we conclude that the presence of the *cit* cluster does enable *E. coli* to multiply independently from carbon and energy sources other than citrate and lactose. Lactose is a necessary co-factor for citrate fermentation in *E. coli* (Lütgens and Gottschalk, 1980). *E. coli* strains that possess the ability to ferment citrate possess a growth advantage relative to *E. coli* that lack the *cit* cluster, because citrate and lactose are abundant in mammary secretions. This might be corroborated by the deregulation observed for genes associated with iron-sulfur cluster biosynthesis, which is also mandatory for citrate metabolism. Accordingly, the deregulation of the expression of the *cit* cluster, in milk whey and milk whey+LPS, reflects a metabolic strategy to utilize citrate, which

is as a major carbon source in mammary secretions and which might be correlated to increased fitness of mastitic *E. coli*. This trait might also enable mastitic *E. coli* to outrun many of the host´s antibacterial defense measures. Recent *in vitro* evolution experiments showed that *E. coli* might gain the ability to use abundant citrate in the growth medium after several thousand generations and thereby were able to reach a much higher population density in this environment (Quandt et al., 2014). In summary, we propose that the ability to utilize citrate could represent a critical bacterial fitness factor of mastitis isolates required for successful intramammary survival.

Role of well-known virulence-associated traits in milk

In the present study, only a very few genes have been identified to be deregulated upon cultivation in milk whey, which could also have a virulence-associated function. These include several outer membrane proteins, or proteins associated with the bacterial membrane. Especially those genes that encode for protein with a leader sequences required for the Sec-dependent secretion pathway are likely to be involved in host-pathogen interaction. Some of them might be therapeutic targets for the development of antibiotics or vaccines. The only gene, with a reported virulence-associated function, which was positively deregulated in milk whey +LPS, was the *tsx* gene. Its gene product is a porin that is presumably involved in different survival strategies to evade immune pressure and to respond to antibiotics. This suggests a significant role as pathogenicity factor (Achouak et al., 2001). The *tsx* gene might be a candidate for further investigation.

VI.1.5. *E. coli* adhesion to cultured mammary gland epithelial cells

This This study clearly demonstrated for the first time that mammary secretions, deficient of cellular components and fat, significantly reduced the ability to adhere to mammary gland epithelial cells. Given the fact that *in vivo* adhesion during mastitis is still questionable, the inhibitory effects on bacterial adhesion have been almost exclusively related to milk fat (globules) and its components (Sánchez-Juanes et al., 2009; Guri et al., 2012; Schroten et al., 1992; Atroshi et al., 1983; Harper et al., 1978). In the present study, it has been demonstrated that rather synergistic effects of mammary secretion compounds account for the inhibition of bacterial adhesion-inhibition than milk fat alone (page 88).

In order to shed light on which non-cellular components contributed to inhibition of *E. coli* cell adhesion, it was required to test different fractions of whole milk for inhibitory components. Assuming that the composition of mammary secretions accounts for growth inhibition, it corroborates the hypothesis of the so called “cow factors”. In contrast, distinct growth characteristics of mastitis isolates *in vitro* have been frequently observed (Döpfer et al., 2000; Dogan et al., 2006; and the present study). Based on their different adhesion and invasion characteristics, it has been hypothesized that *E. coli* strain 1303 differs from isolate ECC-1470 in either its genome content or the

transcriptional activity. Our data indicated that the diverse adhesive properties probably rely on other factors than LpfA, which has been formerly presumed to be an important adherence factor (Dogan et al., 2012). We found that *lpfA* has either been missing in genome of Ec 1303 and was not expressed in *E. coli* ECC-1470. Hence, the colonization of the udder is likely to rely on other factors.

VI.1.6. *E. coli* 1303 and ECC-1470 differed in their gene expression profiles in the presence of mammary gland epithelial cells

The transient mastitis isolate *E. coli* 1303 and the persistent mastitis isolate ECC-1470 differed in their gene expression profiles in presence of mammary gland epithelial cells (**Figure 20**). The analysis of the bacterial transcriptome under conditions mimicking the presence of udder epithelial cells revealed numerous differentially regulated genes relative to the absence of host cells. The identified determinants mainly represent cellular processes that indicate competition for nutrients and growth under anaerobic or at least oxygen-limited conditions. Nevertheless, the description of deregulated gene clusters identified by microarray hybridization requires further confirmation by qRT-PCR.

Competitive growth in presence of mammary gland cells activates stress response

A fraction of the deregulated genes including the Cpx stress response system indicates extracytoplasmic stress. Mastitis *E. coli* isolates may modulate their cell surface characteristics when they encounter host contact. In this context, the Cpx system has been recently reported to potentiate fitness and virulence of ExPEC (Debnath et al., 2013). In contrast, we could neither identify significant deregulation of the *csrA* gene expression, which is involved in the oxidative stress response and host cell invasion (Fields and Thompson, 2008). Instead, expression of the *oxyR* gene was highly up-regulated. OxyR is a major transcriptional regulator required for oxidative stress response. It´s up-regulation was accompanied by up-regulation, although not differentially, of *crp* encoding the cyclic AMP receptor protein Crp. On the one hand Crp is involved in catabolite repression, meaning that as long glucose is available for *E. coli*, the metabolic onversion to other carbohydrate sources is under repression (Deutscher, 2008). Therefore, transcription of catabolic genes involved in energy metabolism is switched on by cAMP-CRP, especially in a setting of cocultivation where epithelial cells and bacteria are competing for glucose. On the other hand it has also been reported that Crp regulate various virulence genes, such as *fim*, *hly*, *tib* (adherence locus) and the *pet* AT toxin-encoding gene, in different *E. coli* pathotypes such as ETEC and UPEC (Müller et al., 2009; Fuentes et al., 2009; Espert et al., 2011; Rossiter et al., 2011; Kansal et al., 2013; Donovan et al., 2013). Furthermore, it is known that *rpoS* might be involved in Crp response(Fuentes et al., 2009). Notably, we found the corresponding gene to be deregulated after three hours of cocultivation of strain ECC-1470 with MAC-T cells. Due to recent findings (Seth et al., 2012), we suggest that this might reflect oxidative or/and nitrosative stress caused by reactive species of either one. However, a presumed regulation of catabolite repression should not be excluded.

The regulators Cpx, CsrA, OxyR, and RpoS belong to a complex regulatory network that has by today not entirely been uncovered. This network comprises cell contact-sensing, signaling cascades and regulation of virulence or fitness-associated factors. Whether the up-regulation of members of this regulatory network is solely reflecting stress response that promotes adaption and survival of *E. coli* under the novel growth conditions or actively contributes to pathogenesis of *E. coli* in the mammary gland requires further investigation.

We can conclude that at least during *in vitro* cocultivation with bovine mammary gland epithelial cells, significantly deregulated genes in both mastitic strains indicate the exposure to extracytoplasmic stress. This is probably caused by metabolic competition for nutrients and the accumulation of metabolic end products of both, bacteria and host cells, in the cell culture medium. Whether this kind of competition also occurs *in vivo* might be questioned, because the mammary gland epithelial cells, which are lining the alveolar space, are supplied by surrounding blood vessels. Given the fact that mainly genes assigned to energy metabolism differed in their expression between the strains, it can be assumed that both strains used different strategies to metabolize under the conditions of cocultivation with MAC-T cells.

Virulence potential of E. coli strains 1303 and ECC 1470 in the presence of host cells

We successfully identified common differential down-regulation of genes related to fimbrial adhesins and flagella. Up-regulated genes involved in protection against host defenses have been observed in both mastitis isolates. Furthermore, a multidrug tolerance system has been up-regulated in both strains. But both mastitis strains also revealed differences in virulence-associated gene expression. The high persistence factor HipA was up-regulated in *E. coli* isolate ECC-1470 suggesting a contribution to the strain's persistence abilities. We also found differentially expressed genes involved in the regulation of curli fimbriae expression. This may contribute to host-pathogen interaction. Further investigations will be necessary to further confirm this correlation between the *E. coli* cell adhesion and/or invasion potential and mastitis pathogenesis.

We hypothesize that there might be correlation between the ability to efficiently adhere to mammary epithelial cells in the presence of milk whey, resistance to the antimicrobial activities of milk whey and an increased mastitis virulence potential. This hypothesis is corroborated by our RNA-Seq-based transcriptome data revealing a marked up-regulation of the expression of determinants involved in the adaptation to growth conditions common in mammary secretions. It might therefore be promising to perform adhesion assays in the presence of milk whey and test different mastitis strains in comparison to faecal isolates.

Whether the different gene expression profiles of *E. coli* strains 1303 and ECC-1470 reflect relevant geno- and phenotypic differences between the pathogenesis of transient and persistent mastitis isolates, or represent strain specific traits, remains to be addressed in the future.

VI.1.7. Peroration / Epilog

Many of the determinants, which we identified to be deregulated under conditions mimicking those during initial stages of mastitis can promote the colonization of extraintestinal niches.

In the past, a putative mammary pathogenic pathotype (MPEC) has been suggested (Shpigel et al., 2008). Based on the data, which we obtained from our phenotypic and transcriptomic analyses, we could rather refuse this suggestion than add further evidence. By employing milk whey and cell culture experiments, the ability to mimic the mammary gland as a niche is a great advantage for understanding the molecular basis of *E. coli* mammary gland infections. We observed that the deregulated bacterial determinants mostly reflected either metabolic adaptation to altered nutritional conditions or adaptation and stress response to environmental conditions. Many of these deregulated determinants cannot be suggested to be mastitis-specific, because they did not reveal any remarkable processes which could be specifically related to either the interaction with host cells or the survival in mammary secretions. Of the determinants that have been differentially expressed in response to intramammary-like conditions, only a few may have the potential to be used as therapeutic targets for the development of antibiotics or vaccines against mastitis *E. coli*. Specific resistance genes against antimicrobial effects of mammary secretion could not be detected.

We anticipate that the definition of a "mammary pathogenic *E. coli*" pathotype that differens from other pathotypes becomes more and more less reasonable. It seems more that mastitis *E. coli* can efficiently sense the presence of particular components of milk whey and respond in a way that they trying to outrun the host response by fast growth.

VI.2. Outlook

In the present study, various determinants involved in adaptation during the transition from the environment into the host have been identified by comparative analysis of differentially expressed genes. These corresponding genes might be located on mobile genetic elements such as genomic islands and prophages that have been recently identified in the genomes of mastitis *E. coli* strains 1303 and ECC-1470 (Dobrindt, unpublished data). If so, this may suggest that specific adaptation, fitness and competitiveness in the intramammary habitat might select for certain mobile genetic elements. Their identification might be useful in therapeutic application and epidemiology.

Regarding our RNA-Seq analyses, the sequencing reads were only mapped to annotated coding sequences. Coding sequences that have not been annotated in the reference genomes, such as intercistronic regions, regions coding for transfer-messenger RNA, regulatory RNAs, such riboswitches, have not been included into our analysis. 10-fold of the amount of reads mapped in the RNA-Seq analysis has not been mapped to the annotated ORFs of our reference genome. Some of these reads might align to intergenic regions or coding sequences that were not or not correctly

annotated. A mapping to the whole reference genome sequence might represent an exciting task also to study differential expression of untranslated coding sequences including regulatory RNAs, etc. . Moreover, it would be interesting to perform a comprehensive alignment and clustering of the unmapped reads to similar sequences of the *E. coli* pangenome. Consequently, this might reveal either one, i) novel expressed protein-coding sequences, or ii) putative non-coding RNAs. The identification of novel non-coding RNAs or riboswitches may be expected. Whether these regulatory elements contribute to the bacterial ability to successfully cause mastitis remains yet unrevealed. Last but not least, the RNA-Seq data obtained can be used to uncover unknown transcriptional start sides as well as antisense transcripts and SNPs in the reference genomes.

Another important task will be to study the in-depth-role of the particular determinants that have been deregulated during either cocultivation with mammary gland epithelial cells or in response to milk whey. Specifically, the impact of citrate utilization should be addressed in comparative growth assays with *E. coli* isolates from bovine mastitis and bovine faeces. This is even more interesting when focusing on comparison to the specific faecal isolates that showed reduced fitness in milk whey (section V.3.1).

Mastitis *E. coli* strains are exposed to selective pressure during growth in the mammary gland. In order to identify determinants that are critical for survival in mammary secretions, a transposon mutagenesis approach will be performed. Therefore, a mastitis *E. coli* transposon mutant library in will be subjected to prolonged growth in milk whey to allow for the enrichment of mutants with improved growth or survival characteristics. As shown in the present study, the antimicrobial effects preserved in milk whey represent an obstacle to bacterial survival. This approach might therefore help to uncover important functional determinants, novel effectors and regulators that are involved in survival in mammary secretions.

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VIII. APPENDIX

VIII.1. List of figures and tables

VIII.2. Transcriptome data

VIII.2.1. Transcriptome data of microarray analyses

Data derived from differential gene expression profiling of mastitis *E. coli* 1303 and ECC-1470 in the presence of MAC-T epithelial cells *in vitro* relative to the absence of cells, sorted according to their level of expression.

Table 18: *E. coli* 1303 up-regulated genes at 1 h cocultivation with MAC-T cells

Gene	Description	Ratio	*p*-value
c3719	Hypothetical protein	5.794	0.000490
c4838	Hypothetical protein	5.646	0.000450
hypF	carbamoyl phosphate phosphatase for [NiFe] hydrogenases	4.671	0.000638
metR	Transcriptional activator protein metR	4.482	0.002368
cpsB	mannose-1-phosphate guanyltransferase	4.297	0.002090
yliL	hypothetical protein	4.199	0.000296
oxyR	DNA-binding transcriptional dual regulator	3.892	0.001078
Z4188	type III secretion apparatus protein	3.569	0.001735
bglF	enzyme; Transport of small molecules: Carbohydrates, organic acids, alcohols	3.312	0.001376
ybaT	predicted transporter	3.308	0.004683
c3354	Hypothetical protein	3.241	0.002973
yeaJ	predicted diguanylate cyclase	3.231	0.002499
yehV	MerR-like regulator A	3.219	0.001612
pppA	putative prepilin peptidase A	3.079	0.002971
L7078	hypothetical protein	3.046	0.000189
c4059	Hypothetical protein	3.024	0.000756
yfaT	hypothetical protein	3.011	0.004204
ECP_3840	putative transposase	2.973	0.000124
c3873	Putative conserved protein	2.931	0.000001
ECs3006	putative C4-type zinc finger protein	2.920	0.000050
hydN	formate dehydrogenase-H, [4Fe-4S] ferredoxin subunit	2.859	0.002034
tauB	transport; Transport of small molecules: Amino acids, amines	2.853	0.001307
c3951	Hypothetical protein	2.848	0.000867
c1956	Putative outer membrane protein yieC precursor	2.793	0.000054
yiaY	predicted Fe-containing alcohol dehydrogenase	2.763	0.000373
c3902	Hypothetical protein	2.748	0.000338
rbn	ribonuclease BN	2.736	0.000960
hycG	hydrogenase 3 and formate hydrogenase complex, HycG subunit	2.649	0.002770
tauA	transport; Transport of small molecules: Amino acids, amines	2.532	0.000280
mdtG	predicted drug efflux system	2.433	0.002238
lldD	L-lactate dehydrogenase, FMN-linked	2.427	0.000035
yigM	predicted inner membrane protein	2.410	0.000634
ycdH	predicted oxidoreductase, flavin: NADH component	2.393	0.000652
caiB	crotonobetainyl-CoA: carnitine CoA-transferase	2.345	0.000442
c1620	Hypothetical protein	2.310	0.000197
htpX	orf; Adaptations, atypical conditions	2.306	0.001447
c4556	Conserved hypothetical protein	2.250	0.000437
c0467	Hypothetical protein yaiO	2.233	0.002603
tsr	methyl-accepting chemotaxis protein I, serine sensor receptor	2.231	0.001665
secA	transport; Protein, peptide secretion	2.225	0.000035
hycH	protein required for maturation of hydrogenase 3	2.203	0.004783
c4942	Hypothetical protein	2.200	0.001512
c5192	Conserved hypothetical protein	2.171	0.000946
hycD	hydrogenase 3, membrane subunit	2.129	0.000051
ygjO	predicted methyltransferase small domain	2.124	0.000688
ybhI	predicted transporter	2.113	0.002998
yjgB	predicted alcohol dehydrogenase, Zn-dependent and NAD(P)-binding	2.042	0.000792
c1463	Hypothetical protein	2.010	0.000361

Gene	Description	Ratio	*p*-value
yciG	hypothetical protein	1.948	0.004322
c1589	putative tail component of prophage	1.937	0.003162
astC	succinylornithine transaminase, PLP-dependent	1.920	0.002398
galS	DNA-binding transcriptional repressor	1.918	0.001912
ygfU	Putative purine permease ygfU	1.876	0.000591
ygeD	predicted inner membrane protein	1.855	0.000222
kdpD	fused sensory histidine kinase in two-component regulatory system with KdpE	1.831	0.001854
ilvL	ilvG operon leader peptide	1.830	0.000804
c0317	Conserved hypothetical protein	1.826	0.000576
hisF	imidazole glycerol phosphate synthase subunit HisF	1.820	0.000268
Z2343	putative outer membrane protein Lom precursor of prophage CP-933O	1.818	0.001498
uidC	predicted outer membrane porin protein	1.772	0.000168
fsaB	fructose-6-phosphate aldolase 2	1.767	0.002446
c1989	Putative acid shock protein	1.762	0.001511
bioB	biotin synthase	1.761	0.000023
umuC	DNA polymerase V subunit UmuC	1.746	0.001525
wcaB	Putative colanic acid biosynthesis acetyltransferase wcaB	1.742	0.002575
gmr	modulator of RNase II stability	1.725	0.003046
yhhW	hypothetical protein	1.714	0.000374
yqhD	alcohol dehydrogenase, NAD(P)-dependent	1.686	0.000390
ybjJ	predicted transporter	1.678	0.000529
cpxP	periplasmic protein combats stress	1.667	0.000372
ytjA	hypothetical protein	1.654	0.000358
c0463	Hypothetical protein	1.645	0.002619
rbsR	DNA-binding transcriptional repressor of ribose metabolism	1.643	0.004083
ymgE	predicted inner membrane protein	1.632	0.000446
metB	cystathionine gamma-synthase	1.617	0.000855
ompW	Outer membrane protein W precursor	1.614	0.000270
UTI89_C5126	putative tail component of prophage CP-933K	1.601	0.001454
ycfA	hypothetical protein	1.559	0.000382
Z3309	putative tail fiber protein encoded within prophage CP-933V	1.545	0.000127
traD	DNA binding protein TraD	1.543	0.003224
yfaZ	Hypothetical protein yfaZ precursor	1.516	0.000941
ECP_4322	multidrug efflux system protein MdtO	1.496	0.003216
flhB	flagellar biosynthesis protein B	1.491	0.000847
malY	bifunctional beta-cystathionase, PLP-dependent/ regulator of maltose regulon	1.486	0.001431
tauB	taurine transporter subunit	1.458	0.000210
ompN	outer membrane pore protein N, non-specific	1.447	0.001406
c4999	Hypothetical protein	1.446	0.001097
menD	enzyme; Biosynthesis of cofactors, carriers: Menaquinone, ubiquinone	1.433	0.000095
yraQ	predicted permease	1.422	0.000250
c3166	putative head-tail joining protein of prophage	1.412	0.001365
aer	fused signal transducer for aerotaxis sensory	1.400	0.004466
aaeX	membrane protein of efflux system	1.374	0.000102
menD	2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase	1.325	0.000397
fliR	Flagellar biosynthetic protein fliR	1.302	0.000315
c1584	putative tail component of prophage | putative tail component of prophage	1.285	0.004092
hyfG	hydrogenase 4, subunit	1.281	0.002185
yegS	hypothetical protein	1.250	0.001462
yihQ	alpha-glucosidase	1.244	0.000783
grxA	glutaredoxin 1, redox coenzyme for ribonucleotide reductase (RNR1a)	1.232	0.000165
sbmC	DNA gyrase inhibitor	1.230	0.000412
c0315	Hypothetical protein	1.227	0.000409
ECP_4339	phosphonates transport ATP-binding protein PhnL	1.224	0.001376
srlA	glucitol/sorbitol-specific enzyme IIC component of PTS	1.219	0.001728
yohL	hypothetical protein	1.194	0.004174
yiiM	hypothetical protein	1.187	0.004260
nhoA	N-hydroxyarylamine O-acetyltransferase	1.183	0.003742
maeB	malic enzyme	1.159	0.004774
ycdS	predicted outer membrane protein	1.157	0.001420
ybbY	predicted uracil/xanthine transporter	1.152	0.000967
aqpZ	aquaporin Z	1.149	0.000040
yhcM	conserved protein with nucleoside triphosphate hydrolase domain	1.147	0.000136

Gene	Description	Ratio	*p*-value
Z3626	sucrose specific transcriptional regulator	1.139	0.004344
ECP_2815	hypothetical protein	1.135	0.000311
ydcU	predicted spermidine/putrescine transporter subunit	1.124	0.002334
ECP_2970	fimbrial usher protein PixC	1.115	0.001174
phnE	phosphonate/organophosphate ester transporter subunit	1.114	0.002051
yidL	predicted DNA-binding transcriptional regulator	1.109	0.004146
baeS	sensory histidine kinase in two-component regulatory system with BaeR	1.104	0.000079
ypdI	Hypothetical lipoprotein ypdI precursor	1.093	0.000074
ubiA	4-hydroxybenzoate octaprenyltransferase	1.077	0.000037
hycC	NADH dehydrogenase subunit N	1.077	0.000301
ECP_0718	hypothetical protein	1.071	0.000450
ubiF	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	1.067	0.000481
mhpC	2-hydroxy-6-ketonona-2,4-dienedioic acid hydrolase	1.046	0.004197
c2987	Ethanolamine utilization protein eutS	1.020	0.000077
ygbF	hypothetical protein	1.019	0.000300
ypdI	predicted lipoprotein involved in colanic acid biosynthesis	1.009	0.000313
ydhP	predicted transporter	1.007	0.000092

Table 19: *E. coli* 1303 down-regulated genes at 1 h cocultivation with MAC-T cells

Gene	Description	Ratio	*p*-value
narZ	nitrate reductase 2 (NRZ), alpha subunit	-1.903	0.003663
ydhY	predicted 4Fe-4S ferridoxin-type protein	-1.760	0.002152
yhhH	hypothetical protein	-1.571	0.004907
Z2101	putative endonuclease encoded within prophage CP-933O	-1.471	0.000275
Z2118	putative endopeptidase Rz of prophage CP-933O	-1.421	0.004356
ycfJ	hypothetical protein	-1.249	0.001798
citG	triphosphoribosyl-dephospho-CoA transferase	-1.243	0.002905
ECP_3800	putative regulatory protein	-1.224	0.002555
yjiX	hypothetical protein	-1.219	0.002110
yjiY	predicted inner membrane protein	-1.188	0.002450
cspA	major cold shock protein	-1.168	0.003196
guaD	guanine deaminase	-1.148	0.000830
yohM	membrane protein conferring nickel and cobalt resistance	-1.120	0.000324
yieL	predicted xylanase	-1.102	0.002387
ydhK	conserved inner membrane protein	-1.079	0.001907
sufC	cysteine desulfurase ATPase component	-1.004	0.000830

Table 20: *E. coli* 1303 up-regulated genes at 3 h cocultivation with MAC-T cells

Gene	Description	Ratio	*p*-value
L7057	replication protein	3.044	0.027619
c4838	Hypothetical protein	3.003	0.023735
ybhI	predicted transporter	2.306	0.007027
oxyR	DNA-binding transcriptional dual regulator	2.218	0.023817
c3719	Hypothetical protein	2.072	0.042827
hypF	carbamoyl phosphate phosphatase [NiFe] hydrogenases	1.985	0.017999
bglF	enzyme; Transport of small molecules: Carbohydrates, organic acids, alcohols	1.975	0.001430
UTI89_C2967	bacteriophage V tail protein	1.851	0.012296
amtB	ammonium transporter	1.820	0.020194
tauB	transport; Transport of small molecules: Amino acids, amines	1.733	0.008654
ydcU	predicted spermidine/putrescine transporter subunit	1.649	0.001534
napA	nitrate reductase, periplasmic, large subunit	1.645	0.025310
gltI	glutamate and aspartate transporter subunit	1.640	0.015068
nuoM	NADH dehydrogenase subunit M	1.606	0.007986
ynfJ	putative voltage-gated ClC-type chloride channel ClcB	1.601	0.023349
ygbI	predicted DNA-binding transcriptional regulator	1.585	0.044468
yfcB	hypothetical protein	1.564	0.003798

Gene	Description	Ratio	*p*-value
nirB	nitrite reductase, large subunit, NAD(P)H-binding	1.549	0.032192
c4556	Conserved hypothetical protein	1.513	0.012680
c0317	Conserved hypothetical protein	1.485	0.001379
c5192	Conserved hypothetical protein	1.483	0.001783
yjgB	predicted alcohol dehydrogenase, Zn-dependent and NAD(P)-binding	1.466	0.000480
UTI89_C3197	ClpB protein	1.461	0.006134
yebU	Hypothetical protein yebU	1.457	0.042089
menD	enzyme; Biosynthesis of cofactors, carriers: Menaquinone, ubiquinone	1.453	0.007420
c2130	Hypothetical protein	1.436	0.032182
yeaG	conserved protein with nucleoside triphosphate hydrolase domain	1.433	0.026552
Z3951	hypothetical protein	1.418	0.049730
ycjB	hypothetical protein	1.416	0.042036
holE	DNA polymerase III, theta subunit	1.413	0.027867
baeS	sensory histidine kinase in two-component regulatory system with BaeR	1.407	0.000517
uidC	predicted outer membrane porin protein	1.398	0.028717
pabA	para-aminobenzoate synthase component II	1.388	0.026282
tauA	transport; Transport of small molecules: Amino acids, amines	1.383	0.021129
poxB	pyruvate dehydrogenase	1.372	0.008276
cysU	sulfate/thiosulfate transporter subunit	1.366	0.032848
hyfF	NADH dehydrogenase subunit N	1.364	0.021507
ubiF	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	1.364	0.010392
yccJ	hypothetical protein	1.363	0.009016
yhhK	hypothetical protein	1.349	0.036169
adhP	enzyme; Energy metabolism, carbon: Anaerobic respiration	1.341	0.043940
deoB	phosphopentomutase	1.332	0.030176
hemG	protoporphyrin oxidase, flavoprotein	1.331	0.023380
motA	flagellar motor protein MotA	1.325	0.037545
ECP_0962	outer membrane protein A	1.321	0.040723
paaB	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation	1.318	0.016941
narV	nitrate reductase 2 (NRZ), gamma subunit	1.313	0.032679
yeiM	predicted nucleoside transporter	1.306	0.032445
asnB	asparagine synthetase B	1.305	0.039901
rpoD	RNA polymerase sigma factor	1.292	0.034000
ycdL	predicted enzyme	1.292	0.008046
ybjJ	predicted transporter	1.284	0.001312
ECP_0718	hypothetical protein	1.277	0.013351
ECP_4773	DNA repair protein RadA	1.274	0.014811
fbaA	fructose-bisphosphate aldolase	1.263	0.039281
ycdX	hypothetical protein	1.257	0.041226
glgA	glycogen synthase	1.244	0.015200
rpmD	50S ribosomal protein L30	1.242	0.039472
appB	cytochrome bd-II oxidase, subunit II	1.240	0.018357
yjbO	phage shock protein G	1.221	0.027524
cfa	cyclopropane fatty acyl phospholipid synthase unsaturated-phospholipid methyltransferase	1.219	0.033955
gyrB	DNA gyrase subunit B	1.215	0.015575
feoB	ferrous iron transport protein B	1.212	0.031098
aqpZ	aquaporin Z	1.202	0.004755
hisB	imidazole glycerol-phosphate	1.200	0.041844
ldcC	Energy metabolism, carbon: Pyruvate dehydrogenase	1.199	0.035472
kdpB	potassium-transporting ATPase subunit B	1.198	0.021652
yeaH	Hypothetical protein yeaH	1.189	0.025011
hycG	hydrogenase 3 and formate hydrogenase complex, HycG subunit	1.187	0.036814
Z1099	hypothetical protein	1.180	0.028565
ompC	outer membrane porin protein C	1.177	0.002938
yqhD	alcohol dehydrogenase, NAD(P)-dependent	1.170	0.010367
ypfG	hypothetical protein	1.166	0.027078
frdA	fumarate reductase	1.164	0.010756
mlrA	DNA-binding transcriptional regulator	1.164	0.007277
dld	D-lactate dehydrogenase, FAD-binding, NADH independent	1.163	0.033742
ygdD	conserved inner membrane protein	1.162	0.016316
hisF	imidazole glycerol phosphate synthase subunit HisF	1.158	0.046363

Gene	Description	Ratio	*p*-value
ydhQ	hypothetical protein	1.156	0.004553
glgB	glycogen branching enzyme	1.154	0.039485
phoP	DNA-binding response regulator in two-component regulatory system with PhoQ	1.154	0.019834
yrbB	hypothetical protein	1.151	0.040928
insF	IS3 element protein InsF	1.150	0.033735
rpsS	30S ribosomal protein S19	1.150	0.042759
paaC	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation	1.149	0.026871
psb1	putative regulator of SOS induction	1.148	0.014526
ygeD	predicted inner membrane protein	1.146	0.041707
insF	IS3 element protein InsF	1.144	0.007049
nhoA	N-hydroxyarylamine O-acetyltransferase	1.144	0.045176
yleB	-	1.140	0.027949
c3164	putative factor; DNA packaging, phage assembly (Phage or Prophage Related)	1.139	0.048254
ompR	osmolarity response regulator	1.136	0.018228
menD	2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase	1.135	0.001747
paaE	predicted multicomponent oxygenase/reductase subunit for phenylacetic acid degradation	1.132	0.033189
phnI	carbon-phosphorus lyase complex subunit	1.132	0.027304
lysU	lysine tRNA synthetase, inducible	1.131	0.047845
ECs5484	hypothetical protein	1.126	0.042031
c3158	putative tail component of prophage	1.123	0.027113
ilvE	branched-chain amino acid aminotransferase	1.120	0.017555
hybF	protein involved with the maturation of hydrogenases 1 and 2	1.117	0.007163
yeaH	hypothetical protein	1.115	0.037149
insF	IS3 element protein InsF	1.114	0.007586
menE	O-succinylbenzoic acid--CoA ligase	1.113	0.025990
pgi	glucose-6-phosphate isomerase	1.112	0.017892
blc	outer membrane lipoprotein (lipocalin)	1.106	0.044586
abrB	AbrB protein (AidB regulator)	1.103	0.004759
nhoA	N-hydroxyarylamine O-acetyltransferase	1.100	0.037641
nlpE	lipoprotein involved with copper homeostasis and adhesion	1.099	0.047162
c2748	Hypothetical protein	1.098	0.015183
prlC	oligopeptidase A	1.098	0.005893
deoA	thymidine phosphorylase	1.097	0.010418
Z0970	putative tail component of prophage CP-933K	1.092	0.023533
dnaJ	chaperone Hsp40, co-chaperone with DnaK	1.092	0.017940
ydfR	hypothetical protein	1.091	0.026310
ygbE	conserved inner membrane protein	1.089	0.034185
yhdY	predicted amino-acid transporter subunit	1.087	0.014403
Z0980	putative tail component of prophage CP-933K	1.086	0.036482
yehW	predicted transporter subunit: membrane component of ABC superfamily	1.084	0.018854
c4837	Hypothetical protein	1.082	0.039611
frdB	fumarate reductase (anaerobic), Fe-S subunit	1.071	0.028053
hyaF	protein involved in nickel incorporation into hydrogenase-1 proteins	1.070	0.006551
narH	enzyme; Energy metabolism, carbon: Anaerobic respiration	1.068	0.014442
cpxP	periplasmic protein combats stress	1.067	0.006446
yhbS	predicted acyltransferase with acyl-CoA N-acyltransferase domain	1.066	0.036482
cyoB	cytochrome o ubiquinol oxidase subunit I	1.061	0.037608
menC	enzyme; Biosynthesis of cofactors, carriers: Menaquinone, ubiquinone	1.060	0.024643
yhfG	hypothetical protein	1.060	0.035312
manY	mannose-specific enzyme IIC component of PTS	1.059	0.043016
yhhW	hypothetical protein	1.051	0.007211
ybeX	predicted ion transport	1.050	0.004095
ydhH	anhydro-N-acetylmuramic acid kinase	1.050	0.048120
yedP	hypothetical protein	1.050	0.035910
gadC	predicted glutamate:gamma-aminobutyric acid antiporter	1.047	0.035811
yjbE	hypothetical protein	1.044	0.002949
ymgG	hypothetical protein	1.044	0.040853
gudD	(D)-glucarate dehydratase 1	1.043	0.046464
moeA	molybdopterin biosynthesis protein	1.042	0.022639
lomR	Rac prophage; predicted protein, N-ter fragment pseudogene	1.041	0.002790

Gene	Description	Ratio	*p*-value
ptsG	fused glucose-specific PTS enzymes: IIB	1.041	0.029970
ECP_0724	hypothetical protein	1.039	0.020607
astB	succinylarginine dihydrolase	1.036	0.015323
ybhC	predicted pectinesterase	1.036	0.023371
asnA	asparagine synthetase AsnA	1.035	0.013957
fbp	fructose-1,6-bisphosphatase	1.035	0.008513
fimC	chaperone, periplasmic	1.035	0.027329
ybeY	hypothetical protein	1.031	0.006340
sufA	iron-sulfur cluster assembly scaffold protein	1.028	0.037932
gudP	predicted D-glucarate transporter	1.027	0.039662
Z4045	hypothetical protein	1.021	0.040670
ubiA	4-hydroxybenzoate octaprenyltransferase	1.020	0.000353
rpmJ	50S ribosomal protein L36	1.018	0.034127
torY	TMAO reductase III (TorYZ), cytochrome c-type subunit	1.018	0.033727
frdC	fumarate reductase subunit C	1.015	0.027717
yfeH	predicted inner membrane protein	1.013	0.015005
rnr	exoribonuclease R, RNase R	1.011	0.023250
wrbA	TrpR binding protein WrbA	1.011	0.018289
hyaA	hydrogenase 1, small subunit	1.007	0.005126
cadA	lysine decarboxylase 1	1.006	0.018937
rpoS	RNA polymerase sigma factor	1.005	0.021264
c1462	putative factor; DNA packaging, phage assembly (Phage or Prophage Related)	1.004	0.047156
ldcC	positive control	1.004	0.043481
yggR	predicted transporter	1.004	0.026766
cbpM	modulator of CbpA co-chaperone	1.002	0.010852
ybhQ	predicted inner membrane protein	1.001	0.031647

Table 21: *E. coli* 1303 down-regulated genes at 3 h cocultivation with MAC-T cells

Gene	Description	Ratio	*p*-value
c5000	Hypothetical protein	-3.647	0.027907
Z2101	putative endonuclease encoded within prophage CP-933O	-3.546	0.024686
yddA	fused predicted multidrug transporter subunits	-2.946	0.019734
torS	enzyme; Energy metabolism, carbon: Anaerobic respiration	-2.789	0.040084
yibD	predicted glycosyl transferase	-2.384	0.010253
yjaI	Zinc resistance-associated protein precursor	-2.383	0.036959
malF	maltose transporter subunit	-2.260	0.006608
cynT	carbonic anhydrase	-2.238	0.039457
c3219	Hypothetical protein	-2.185	0.041744
yehR	hypothetical protein	-2.131	0.013630
cusC	copper/silver efflux system, outer membrane component	-2.084	0.045023
cspG	DNA-binding transcriptional regulator	-2.075	0.038116
c0670	Hypothetical protein	-2.038	0.046628
yfgI	hypothetical protein	-2.026	0.047741
flgC	flagellar basal-body rod protein C	-1.978	0.049963
ydhI	predicted inner membrane protein	-1.860	0.045241
narY	nitrate reductase 2 (NRZ), beta subunit	-1.826	0.047165
tnaB	tryptophan transporter of low affinity	-1.714	0.032191
sfmA	predicted fimbrial-like adhesin protein	-1.697	0.005352
dos	cAMP phosphodiesterase, heme-regulated	-1.673	0.042243
trpD	Anthranilate synthase component II	-1.656	0.042104
ycgF	Hypothetical protein ycgF	-1.655	0.042258
ycfJ	hypothetical protein	-1.631	0.024190
yliE	conserved inner membrane protein	-1.631	0.034862
tdcA	DNA-binding transcriptional activator	-1.614	0.030906
mgtA	magnesium transporter	-1.610	0.032254
ybcS	DLP12 prophage; predicted lysozyme	-1.599	0.044947
yhcC	predicted Fe-S oxidoreductase	-1.577	0.048241
torC	enzyme; Energy metabolism, carbon: Anaerobic respiration	-1.503	0.047567
yaiS	hypothetical protein	-1.464	0.024833
ytfQ	predicted sugar transporter subunit: periplasmic-binding component of ABC	-1.459	0.047644

Gene	Description	Ratio	*p*-value
	superfamily		
yidI	predicted inner membrane protein	-1.453	0.034156
ycaO	hypothetical protein	-1.443	0.020393
c3831	Hypothetical protein	-1.415	0.018194
yoaG	hypothetical protein	-1.415	0.044636
L7046	ORFB of IS911	-1.393	0.021846
ulaC	L-ascorbate-specific enzyme IIA component of PTS	-1.371	0.006725
suhB	inositol monophosphatase	-1.369	0.026031
c5233	Hypothetical protein	-1.362	0.045485
dgoD	galactonate dehydratase	-1.361	0.041268
yciW	predicted oxidoreductase	-1.360	0.001883
phnH	carbon-phosphorus lyase complex subunit	-1.348	0.001862
emrB	multidrug efflux system protein	-1.336	0.019879
pinR	Rac prophage; predicted site-specific recombinase	-1.318	0.040083
Z1627	hypothetical protein	-1.309	0.040993
yiaM	predicted transporter	-1.283	0.035292
Z4360	hypothetical protein	-1.280	0.030250
ycdQ	predicted glycosyl transferase	-1.277	0.003520
dinI	DNA damage-inducible protein I	-1.273	0.036503
trbE	inner membrane protein TrbE	-1.269	0.031204
narY	nitrate reductase 2 (NRZ), beta subunit	-1.264	0.022200
xdhD	fused predicted xanthine/hypoxanthine oxidase:	-1.258	0.029038
Z2343	putative outer membrane protein Lom precursor of prophage CP-933O	-1.242	0.043015
yfiP	hypothetical protein	-1.242	0.032926
ydeP	predicted oxidoreductase	-1.201	0.024745
ybdN	hypothetical protein	-1.200	0.037550
yigN	DNA recombination protein rmuC	-1.194	0.045339
fadL	long-chain fatty acid outer membrane transporter	-1.192	0.009722
c1824	Hypothetical protein	-1.171	0.029865
yfcT	predicted outer membrane export usher protein	-1.157	0.041684
yhhQ	conserved inner membrane protein	-1.156	0.014234
yeaA	hypothetical protein	-1.153	0.019970
ygjJ	hypothetical protein	-1.150	0.034512
ydiR	predicted electron transfer flavoprotein, FAD-binding	-1.145	0.029607
pinQ	Qin prophage; predicted site-specific recombinase	-1.141	0.023897
cheR	chemotaxis regulator, protein-glutamate methyltransferase	-1.139	0.002576
ybdL	putative aminotransferase	-1.131	0.008954
yncC	predicted DNA-binding transcriptional regulator	-1.130	0.019183
c2318	Hypothetical protein	-1.116	0.012856
flgN	export chaperone for FlgK and FlgL	-1.110	0.036724
ydiE	Hypothetical protein ydiE	-1.108	0.012708
fliS	flagellar protein FliS	-1.099	0.003092
yfeD	predicted DNA-binding transcriptional regulator	-1.085	0.045178
narZ	nitrate reductase 2 (NRZ), alpha subunit	-1.084	0.034313
ybfC	hypothetical protein	-1.068	0.003355
insB	CP4-6 prophage; IS1 transposase InsAB'	-1.067	0.022609
tnaA	tryptophanase/L-cysteine desulfhydrase, PLP-dependent	-1.064	0.001795
ypeC	hypothetical protein	-1.062	0.045315
phoA	bacterial alkaline phosphatase	-1.023	0.013970
c4140	Hypothetical protein	-1.021	0.002267
uxaB	tagaturonate reductase	-1.019	0.048286
ygeV	predicted DNA-binding transcriptional regulator	-1.017	0.035001
yedQ	predicted diguanylate cyclase	-1.009	0.017396
ycjQ	Hypothetical zinc-type alcohol dehydrogenase-like protein ycjQ	-1.008	0.048391

Table 22: ECC-1470 up-regulated genes at 1 h cocultivation with MAC-T cells

Gene	Description	Ratio	*p*-value
L7057	replication protein	6.538	0.000309
hypF	carbamoyl phosphate phosphatase and maturation protein for [NiFe] hydrogenases	5.844	0.000428

Gene	Description	Ratio	*p*-value
hycG	hydrogenase 3 and formate hydrogenase complex, HycG subunit	5.227	0.000465
bglF	enzyme; Transport of small molecules: Carbohydrates, organic acids, alcohols	4.922	0.000270
c3719	Hypothetical protein	4.824	0.001407
metR	Transcriptional activator protein metR	4.619	0.000164
tauB	transport; Transport of small molecules: Amino acids, amines	4.244	0.000166
nfrA	bacteriophage N4 receptor, outer membrane subunit	4.192	0.004571
molR	-	4.152	0.008823
gsp	bifunctional glutathionylspermidine amidase/glutathionylspermidine synthetase	4.080	0.002106
ybaT	predicted transporter	3.942	0.002652
yfaT	hypothetical protein	3.889	0.001320
ygjD	Probable O-sialoglycoprotein endopeptidase	3.751	0.004062
c1620	Hypothetical protein	3.727	0.001073
oxyR	DNA-binding transcriptional dual regulator	3.633	0.004445
c3354	Hypothetical protein	3.588	0.013434
yliL	hypothetical protein	3.531	0.002085
c3549	-	3.528	0.017049
c1584	putative tail component of prophage | putative tail component of prophage	3.510	0.004948
c3902	Hypothetical protein	3.494	0.005234
yjgB	predicted alcohol dehydrogenase, Zn-dependent and NAD(P)-binding	3.489	0.003475
ppdA	hypothetical protein	3.479	0.003744
c4942	Hypothetical protein	3.458	0.004752
ygjO	predicted methyltransferase small domain	3.423	0.002768
c4303	Putative conserved protein	3.407	0.007162
c1956	Putative outer membrane protein yieC precursor	3.362	0.001255
yiaY	predicted Fe-containing alcohol dehydrogenase	3.325	0.000864
mdtG	predicted drug efflux system	3.323	0.002465
yehV	MerR-like regulator A	3.274	0.018437
ycdH	predicted oxidoreductase, flavin:NADH component	3.218	0.001378
hydN	formate dehydrogenase-H, [4Fe-4S] ferredoxin subunit	3.168	0.005405
c0703	Hypothetical protein	3.121	0.002688
c4250	Hypothetical protein	3.032	0.040438
ECP_3840	putative transposase	3.000	0.001275
wcaB	Putative colanic acid biosynthesis acetyltransferase wcaB	2.919	0.000674
yiaO	Putative ABC transporter periplasmic binding protein yiaO precursor	2.883	0.008031
Z3309	putative tail fiber protein of prophage CP-933V	2.851	0.004688
yfaZ	Hypothetical protein yfaZ precursor	2.804	0.031826
UTI89_C5126	putative tail component of prophage CP-933K	2.791	0.002948
tauA	transport; Transport of small molecules: Amino acids, amines	2.785	0.000705
hycD	hydrogenase 3, membrane subunit	2.730	0.009915
c4059	Hypothetical protein	2.724	0.000994
ECs3006	putative C4-type zinc finger protein	2.722	0.001439
Z4188	type III secretion apparatus protein	2.701	0.000710
ymgE	predicted inner membrane protein	2.692	0.000237
astC	succinylornithine transaminase, PLP-dependent	2.644	0.001769
holE	DNA polymerase III, theta subunit	2.626	0.002266
c3873	Putative conserved protein	2.611	0.018204
ygeZ	dihydropyrimidinase	2.611	0.005451
ybhI	predicted transporter	2.596	0.001574
c5192	Conserved hypothetical protein	2.537	0.000388
uidC	predicted outer membrane porin protein	2.535	0.001451
c1463	Hypothetical protein	2.524	0.000459
c0317	Conserved hypothetical protein	2.519	0.000515
ycdC	predicted DNA-binding transcriptional regulator	2.515	0.001495
ompN	outer membrane pore protein N, non-specific	2.436	0.002921
yqhD	alcohol dehydrogenase, NAD(P)-dependent	2.424	0.000896
menD	enzyme; Biosynthesis of cofactors, carriers: Menaquinone, ubiquinone	2.376	0.003283
yjfC	predicted synthetase/amidase	2.371	0.001891
caiB	crotonobetainyl-CoA:carnitineCoA-transferase	2.354	0.002784
yhhW	hypothetical protein	2.344	0.004952
ydcU	predicted spermidine/putrescine transporter subunit	2.337	0.011795
c1589	putative tail component of prophage	2.314	0.003065
menD	2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase	2.280	0.003246
c4556	Conserved hypothetical protein	2.269	0.002831

Gene	Description	Ratio	*p*-value
L7078	hypothetical protein	2.207	0.041672
ycfA	hypothetical protein	2.152	0.000461
rbsR	DNA-binding transcriptional repressor of ribose metabolism	2.101	0.002637
ECP_3061	hypothetical protein	2.094	0.008766
yihQ	alpha-glucosidase	2.091	0.005240
c0784	Hypothetical protein	2.026	0.032363
glcD	glycolate oxidase subunit, FAD-linked	2.008	0.006334
sgbH	3-keto-L-gulonate 6-phosphate decarboxylase	1.989	0.008983
baeS	sensory histidine kinase in two-component regulatory system with BaeR	1.957	0.001856
hyfI	hydrogenase 4, Fe-S subunit	1.949	0.016407
hycH	protein required for maturation of hydrogenase 3	1.925	0.003074
ygjG	Probable ornithine aminotransferase | Hypothetical protein	1.921	0.000881
c3164	putative factor; DNA packaging, phage assembly (Phage or Prophage Related)	1.900	0.004407
c0463	Hypothetical protein	1.885	0.020708
mdtI	multidrug efflux system transporter	1.866	0.006324
yraQ	predicted permease	1.860	0.007672
c1109	Hypothetical protein	1.851	0.002810
ygcP	predicted anti-terminator regulatory protein	1.831	0.039243
umuC	DNA polymerase V subunit UmuC	1.828	0.027895
c3166	putative head-tail joining protein of prophage	1.809	0.007384
yaaI	hypothetical protein	1.782	0.002347
ypdI	predicted lipoprotein involved in colanic acid biosynthesis	1.771	0.002269
cpxP	periplasmic protein combats stress	1.770	0.002871
mlrA	DNA-binding transcriptional regulator	1.768	0.000863
yjbO	phage shock protein G	1.764	0.001199
tauB	taurine transporter subunit	1.743	0.000183
c0467	Hypothetical protein yaiO	1.737	0.003283
hipA	regulator; Murein sacculus, peptidoglycan	1.712	0.016415
arcA	DNA-binding response regulator in two-component regulatory system with ArcB or CpxA	1.695	0.044330
insF	IS3 element protein InsF	1.693	0.046772
ECP_2815	hypothetical protein	1.691	0.002401
insF	IS3 element protein InsF	1.684	0.022644
yhaR	hypothetical protein	1.682	0.023635
rbn	ribonuclease BN	1.673	0.039971
yigE	hypothetical protein	1.672	0.034821
lldD	L-lactate dehydrogenase, FMN-linked	1.660	0.043321
yhdX	predicted amino-acid transporter subunit	1.653	0.012202
yidL	predicted DNA-binding transcriptional regulator	1.649	0.002750
kdpD	fused sensory histidine kinase in two-component regulatory system with KdpE: signal sensing protein	1.640	0.017473
yjgH	predicted mRNA endoribonuclease	1.640	0.008030
ilvL	ilvG operon leader peptide	1.621	0.019819
yaaI	Hypothetical protein yaaI precursor	1.619	0.048608
insF	IS3 element protein InsF	1.611	0.037185
klcA	KlcA	1.608	0.030307
ygjQ	Hypothetical protein ygjQ	1.606	0.007918
ypdI	Hypothetical lipoprotein ypdI precursor	1.602	0.000014
ydiY	hypothetical protein	1.588	0.005281
nuoM	NADH dehydrogenase subunit M	1.582	0.002840
flhB	flagellar biosynthesis protein B	1.569	0.017055
potH	putrescine transporter subunit: membrane component of ABC superfamily	1.563	0.048324
hyfG	hydrogenase 4, subunit	1.548	0.016458
hyaA	hydrogenase 1, small subunit	1.534	0.005247
sbmC	DNA gyrase inhibitor	1.531	0.023853
argD	bifunctional acetylornithine aminotransferase/ succinyldiaminopimelate aminotransferase	1.524	0.010162
ECP_2970	fimbrial usher protein PixC	1.519	0.004684
ybjJ	predicted transporter	1.506	0.032003
Z2371	putative lysozyme R of prophage CP-933R	1.505	0.001012
mutH	DNA mismatch repair protein	1.495	0.040984
c0315	Hypothetical protein	1.490	0.020204
nikE	ATP-binding protein of nickel transport system	1.488	0.021870

Gene	Description	Ratio	*p*-value
malY	bifunctional beta-cystathionase, PLP-dependent/ regulator of maltose regulon	1.487	0.000233
yahC	predicted inner membrane protein	1.464	0.000360
ECP_4322	multidrug efflux system protein MdtO	1.457	0.005585
ygfU	Putative purine permease ygfU	1.456	0.011685
srmB	ATP-dependent RNA helicase	1.445	0.004211
murI	glutamate racemase	1.439	0.024540
yihM	Hypothetical protein yihM	1.435	0.040718
murI	glutamate racemase	1.427	0.025648
ymgG	hypothetical protein	1.423	0.003612
nhoA	N-hydroxyarylamine O-acetyltransferase	1.420	0.003969
ygeD	predicted inner membrane protein	1.419	0.023052
ubiF	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	1.418	0.016729
yegS	hypothetical protein	1.414	0.005603
artM	arginine transporter subunit	1.398	0.006017
astB	succinylarginine dihydrolase	1.385	0.040620
crp	DNA-binding transcriptional dual regulator	1.383	0.018503
artM	transport; Transport of small molecules: Amino acids, amines	1.379	0.003453
gamW	putative factor; Integration, recombination (Phage or Prophage Related)	1.375	0.019589
yfcS	putative fimbrial chaperone	1.369	0.023554
yciG	hypothetical protein	1.361	0.000820
yfcB	hypothetical protein	1.360	0.000657
insF	IS3 element protein InsF	1.352	0.032161
insF	IS3 element protein InsF	1.350	0.029820
yeiM	predicted nucleoside transporter	1.339	0.006366
lsrC	AI2 transporter	1.333	0.003747
nhoA	N-hydroxyarylamine O-acetyltransferase	1.333	0.002624
c1036	Hypothetical protein	1.332	0.003587
c2987	Ethanolamine utilization protein eutS	1.326	0.002779
ymgD	hypothetical protein	1.324	0.005204
ydjR	hypothetical protein	1.300	0.007177
kdpB	potassium-transporting ATPase subunit B	1.294	0.004052
ydeD	hypothetical protein	1.293	0.017149
c2440	Hypothetical protein	1.278	0.002465
chbA	N,N'-diacetylchitobiose-specific enzyme IIA component of PTS	1.275	0.015801
yohL	hypothetical protein	1.264	0.007885
yebB	hypothetical protein	1.263	0.010261
UTI89_C1520	hypothetical protein	1.258	0.001566
c5221	Hypothetical protein	1.247	0.000370
UTI89_C2967	bacteriophage V tail protein	1.246	0.005864
ydgI	predicted arginine/ornithine antiporter transporter	1.244	0.007824
fixX	predicted 4Fe-4S ferredoxin-type protein	1.232	0.000799
ECs5296	hypothetical protein	1.229	0.017066
mdtL	multidrug efflux system protein	1.228	0.002782
yebK	predicted DNA-binding transcriptional regulator	1.223	0.044433
flhE	hypothetical protein	1.212	0.008942
Z0415	putative periplasmic binding protein, probable substrate ribose	1.207	0.007037
ybjG	undecaprenyl pyrophosphate phosphatase	1.206	0.039326
hyfF	NADH dehydrogenase subunit N	1.205	0.000394
yfeW	hypothetical protein	1.205	0.012251
ivbL	ilvB operon leader peptide	1.204	0.008047
rhsD	rhsD element protein	1.204	0.013136
ECs0808	hypothetical protein	1.199	0.028575
dppC	dipeptide transporter	1.199	0.011236
argB	Acetylglutamate kinase	1.179	0.001893
UTI89_C2969	bacteriophage V tail/DNA circulation protein	1.178	0.003561
ycjB	hypothetical protein	1.178	0.018251
ssuC	alkanesulfonate transporter subunit	1.168	0.046501
xdhB	xanthine dehydrogenase, FAD-binding subunit	1.164	0.041121
nikB	nickel transporter subunit	1.160	0.001111
yphB	hypothetical protein	1.159	0.002899
yeeF	Hypothetical transport protein yeeF	1.150	0.008800
ybbI	putative transcriptional regulator	1.149	0.023045
traD	DNA binding protein TraD	1.148	0.016531

Gene	Description	Ratio	*p*-value
citE	enzyme; Central intermediary metabolism: Pool, multipurpose conversions	1.137	0.011713
ddpC	D-ala-D-ala transporter subunit	1.136	0.003102
rluA	pseudouridine synthase for 23S rRNA (position 746) and tRNAphe(position 32)	1.135	0.000834
suhB	inositol monophosphatase	1.133	0.003198
fis	DNA-binding protein Fis	1.132	0.029532
fadH	2,4-dienoyl-CoA reductase, NADH and FMN-linked	1.129	0.003623
ygfQ	predicted transporter	1.126	0.008347
UTI89_C3197	ClpB protein	1.122	0.002514
mokB	regulatory peptide | "toxic polypeptide, small "	1.120	0.020108
ECs0727	KdpF protein of high-affinity potassium transport system	1.119	0.006816
ybcC	DLP12 prophage; predicted exonuclease	1.117	0.001304
gpmB	phosphoglycerate mutase	1.116	0.010526
mglB	methyl-galactoside transporter subunit	1.113	0.008778
gatB	galactitol-specific enzyme IIB component of PTS	1.111	0.008108
yhaH	predicted inner membrane protein	1.109	0.048315
yjaB	predicted acetyltransferase	1.105	0.001774
hycC	NADH dehydrogenase subunit N	1.103	0.016189
Z0980	putative tail component of prophage CP-933K	1.095	0.007918
yeaN	predicted transporter	1.094	0.023882
c4837	Hypothetical protein	1.093	0.004382
napA	nitrate reductase, periplasmic, large subunit	1.092	0.000868
kefA	fused conserved protein	1.083	0.003683
artP	arginine transporter subunit	1.080	0.026064
Z0976	putative tail component of prophage CP-933K	1.079	0.005703
eutJ	predicted chaperonin, ethanolamine utilization protein	1.079	0.020978
argD	Acetylornithine aminotransferase	1.078	0.005165
htpX	orf; Adaptations, atypical conditions	1.073	0.042162
cysE	Serine acetyltransferase	1.070	0.017315
Z4186	putative integral membrane protein-component of typeIII secretion apparatus	1.068	0.001604
nlpE	lipoprotein involved with copper homeostasis and adhesion	1.063	0.004385
yhcM	conserved protein with nucleoside triphosphate hydrolase domain	1.062	0.042335
deoR	DNA-binding transcriptional repressor	1.059	0.014503
aer	fused signal transducer for aerotaxis sensory	1.058	0.018864
lysP	lysine transporter	1.058	0.002089
rffA	TDP-4-oxo-6-deoxy-D-glucose transaminase	1.057	0.003916
potI	putrescine transporter subunit: membrane component of ABC superfamily	1.055	0.010077
yhhX	predicted oxidoreductase with NAD(P)-binding Rossmann-fold domain	1.055	0.032840
moaD	molybdopterin synthase, small subunit	1.054	0.007634
ubiA	4-hydroxybenzoate octaprenyltransferase	1.053	0.002326
yaeQ	hypothetical protein	1.050	0.005008
suhB	inositol monophosphatase	1.049	0.008531
ypfE	predicted carboxysome structural protein with predicted role in ethanol utilization	1.049	0.000463
fecD	KpLE2 phage-like element; iron-dicitrate transporter subunit	1.043	0.009066
ECP_0718	hypothetical protein	1.036	0.021118
yeaS	neutral amino-acid efflux system	1.034	0.008381
yehD	Hypothetical protein yehD precursor	1.027	0.002433
c1368	Hypothetical protein | Hypothetical protein	1.026	0.001625
c1846	Putative conserved protein	1.014	0.012133
yabI	conserved inner membrane protein	1.012	0.017029
phnO	predicted acyltransferase with acyl-CoA N-acyltransferase domain	1.010	0.022996
mdaB	NADPH quinone reductase	1.009	0.014804

Table 23: ECC-1470 down-regulated genes at 1 h cocultivation with MAC-T cells

Gene	Description	Ratio	*p*-value
yjgG	hypothetical protein	-4.775	0.010795
citC	citrate lyase synthetase	-4.400	0.035696
glvB	arbutin specific enzyme IIB component of PTS	-3.902	0.007404
ybiM	Hypothetical protein ybiM	-3.858	0.003070
yaiT	-	-3.733	0.040758

Gene	Description	Ratio	*p*-value
ybeQ	hypothetical protein	-3.690	0.040218
ybeR	hypothetical protein	-3.529	0.002737
ydfK	Qin prophage; predicted DNA-binding transcriptional regulator | Rac prophage; predicted DNA-binding transcriptional regulator	-3.352	0.011855
ygfO	predicted transporter	-3.154	0.002954
c3915	Hypothetical protein	-3.116	0.004858
araD	L-ribulose-5-phosphate 4-epimerase	-3.056	0.002394
yibG	hypothetical protein	-2.976	0.039470
yccE	hypothetical protein	-2.906	0.005079
Z3104	putative endolysin of prophage CP-933U	-2.894	0.002457
citD	citrate lyase, acyl carrier (gamma) subunit	-2.869	0.001104
phnH	carbon-phosphorus lyase complex subunit	-2.838	0.025851
ygeX	diaminopropionate ammonia-lyase	-2.745	0.047480
yadD	predicted transposase	-2.713	0.018260
Z5489	hypothetical protein	-2.615	0.045145
ycaM	predicted transporter	-2.600	0.002487
ypeA	Hypothetical protein ypeA	-2.461	0.004545
rspB	predicted oxidoreductase, Zn-dependent and NAD(P)-binding	-2.395	0.013347
ycaC	predicted hydrolase	-2.391	0.001508
csgF	predicted transport protein	-2.356	0.000741
ydiF	fused predicted acetyl-CoA:acetoacetyl-CoA	-2.349	0.005614
yibJ	predicted Rhs-family protein	-2.342	0.008111
-	-	-2.314	0.023797
ygaQ	hypothetical protein	-2.294	0.015992
ydcC	hypothetical protein	-2.253	0.020139
lsrA	fused AI2 transporter subunits of ABC superfamily: ATP-binding components	-2.251	0.002760
yahF	predicted acyl-CoA synthetase with NAD(P)-binding domain and succinyl-CoA synthetase domain	-2.232	0.002067
dgoD	galactonate dehydratase	-2.206	0.000475
ygfK	predicted oxidoreductase, Fe-S subunit	-2.164	0.023550
ygeW	hypothetical protein	-2.152	0.040243
ybiW	predicted pyruvate formate lyase	-2.052	0.008228
stfR	Rac prophage; predicted tail fiber protein	-2.031	0.010701
yddV	predicted diguanylate cyclase	-2.025	0.047055
c1955	6-phospho-beta-glucosidase bglA	-1.979	0.019067
ydhV	predicted oxidoreductase	-1.968	0.039711
arpB	-	-1.963	0.004391
fixB	predicted electron transfer flavoprotein,	-1.915	0.037769
c3841	Hypothetical protein	-1.881	0.033211
ycdT	predicted diguanylate cyclase	-1.830	0.030914
yqeJ	hypothetical protein	-1.819	0.024178
ykgB	conserved inner membrane protein	-1.810	0.003069
htrC	heat shock protein	-1.808	0.028469
phnE	phosphonate/organophosphate ester transporter pseudogene)	-1.797	0.008906
bglF	fused beta-glucoside-specific PTS enzymes: IIA	-1.789	0.012194
rbsA	fused D-ribose transporter subunits of ABC superfamily: ATP-binding components	-1.781	0.018725
phnG	carbon-phosphorus lyase complex subunit	-1.762	0.040161
yjcC	predicted signal transduction protein (EAL domain containing protein)	-1.757	0.003271
yjfF	predicted sugar transporter subunit: membrane component of ABC superfamily	-1.740	0.008509
rarA	predicted hydrolase	-1.722	0.022445
c3163	putative tail component of prophage	-1.681	0.040005
ykgK	predicted regulator	-1.658	0.025115
ybfL	predicted transposase (pseudogene)	-1.656	0.017117
citG	triphosphoribosyl-dephospho-CoA transferase	-1.645	0.033387
yedP	hypothetical protein yedP	-1.639	0.034703
xdhA	xanthine dehydrogenase, molybdenum binding subunit	-1.638	0.015282
yqeA	predicted amino acid kinase	-1.631	0.000686
yzgL	hypothetical protein	-1.621	0.019928
c0697	-	-1.596	0.037141
yicO	predicted xanthine/uracil permease	-1.578	0.012919
yqeI	predicted transcriptional regulator	-1.571	0.000181
Z3956	hypothetical protein	-1.567	0.025559

Gene	Description	Ratio	*p*-value
guaD	guanine deaminase	-1.567	0.007993
hofB	conserved protein with nucleoside triphosphate hydrolase domain	-1.560	0.008706
ygeI	hypothetical protein	-1.555	0.004821
yibI	predicted inner membrane protein	-1.535	0.010711
ylbH	conserved protein, rhs-like	-1.529	0.023590
cusC	copper/silver efflux system, outer membrane component	-1.518	0.010628
recE	Exodeoxyribonuclease VIII	-1.513	0.000905
ybfC	hypothetical protein	-1.513	0.020506
-	-	-1.503	0.042245
dmsC	dimethyl sulfoxide reductase, anaerobic, subunit C	-1.501	0.016040
lacZ	beta-D-galactosidase	-1.491	0.000209
ygfJ	hypothetical protein	-1.491	0.001366
ydhW	hypothetical protein	-1.482	0.011972
ycbJ	hypothetical protein	-1.479	0.006475
yacH	hypothetical protein	-1.467	0.006206
ybbD	hypothetical protein	-1.450	0.030979
csgD	DNA-binding transcriptional activator in two-component regulatory system	-1.420	0.027703
acs	acetyl-coenzyme A synthetase	-1.419	0.018361
ECs1172	hypothetical protein	-1.413	0.009671
phnD	phosphonate/organophosphate ester transporter subunit	-1.413	0.032096
ybhP	predicted DNase	-1.400	0.008249
gadE	DNA-binding transcriptional activator	-1.394	0.034200
ycgX	hypothetical protein	-1.390	0.016818
eutG	predicted alcohol dehydrogenase in ethanolamine utilization	-1.370	0.007222
c2118	Putative conserved protein	-1.363	0.013760
ycaP	conserved inner membrane protein	-1.358	0.000027
yqiI	hypothetical protein	-1.355	0.031472
c2257	Hypothetical protein	-1.354	0.016817
ytfA	predicted transcriptional regulator	-1.352	0.013852
ycfT	predicted inner membrane protein	-1.341	0.043545
yeaH	Hypothetical protein yeaH	-1.328	0.030154
c0446	Hypothetical protein	-1.326	0.032283
sfmA	predicted fimbrial-like adhesin protein	-1.308	0.018815
hyaD	protein involved in processing of HyaA and HyaB proteins	-1.304	0.002184
yncC	predicted DNA-binding transcriptional regulator	-1.304	0.011955
yfgH	predicted outer membrane lipoprotein	-1.290	0.035459
ybiA	hypothetical protein	-1.286	0.017401
yedK	Hypothetical protein yedK	-1.269	0.000844
yehZ	Hypothetical protein yehZ precursor	-1.265	0.005711
c1465	putative factor; DNA packaging, phage assembly (Phage or Prophage Related)	-1.243	0.041976
Z1687	hypothetical protein	-1.235	0.000994
vgrE	unknown protein associated with Rhs element	-1.227	0.003164
tnaC	tryptophanase leader peptide	-1.220	0.007233
yhiX	Transcriptional regulator gadX	-1.217	0.031439
gadW	DNA-binding transcriptional activator	-1.216	0.021935
Z5430	hypothetical protein	-1.215	0.023973
rusA	DLP12 prophage; endonuclease RUS	-1.210	0.009166
ydhY	predicted 4Fe-4S ferridoxin-type protein	-1.209	0.009199
hlyE	hemolysin E	-1.207	0.036128
melA	alpha-galactosidase, NAD(P)-binding	-1.202	0.004292
nirD	enzyme; Energy metabolism, carbon: Anaerobic respiration	-1.201	0.026541
ysaB	hypothetical protein	-1.192	0.025419
ydfO	Qin prophage; predicted protein	-1.190	0.048916
yeaH	hypothetical protein	-1.184	0.024511
yadE	predicted polysaccharide deacetylase lipoprotein	-1.182	0.042611
ydiQ	hypothetical protein	-1.181	0.019205
yfeG	predicted DNA-binding transcriptional regulator	-1.179	0.018008
Z5852	hypothetical protein	-1.178	0.033637
hyfB	NADH dehydrogenase subunit N	-1.178	0.034393
ybhJ	Hypothetical protein ybhJ	-1.169	0.043826
yidI	predicted inner membrane protein	-1.169	0.000487
ybcF	predicted carbamate kinase	-1.154	0.028872
yphH	predicted DNA-binding transcriptional regulator	-1.153	0.038796

Gene	Description	Ratio	*p*-value
yaaJ	predicted transporter	-1.151	0.040073
yhhI	predicted transposase	-1.151	0.001580
melR	DNA-binding transcriptional dual regulator	-1.145	0.037517
ECs5537	hypothetical protein	-1.139	0.021976
c4010	Hypothetical protein	-1.137	0.034508
crcA	palmitoyl transferase for Lipid A	-1.135	0.029276
ykgC	pyridine nucleotide-disulfide oxidoreductase	-1.124	0.006169
ydaM	predicted diguanylate cyclase, GGDEF domain signalling protein	-1.119	0.016123
c0640	Conserved hypothetical protein	-1.117	0.019244
ygjV	conserved inner membrane protein	-1.115	0.004444
mhpR	DNA-binding transcriptional activator, 3HPP-binding	-1.112	0.010268
ybdM	hypothetical protein	-1.112	0.013886
ydiM	predicted transporter	-1.105	0.012265
yebN	conserved inner membrane protein	-1.105	0.034131
c4175	Conserved hypothetical protein	-1.102	0.032028
ycgF	Hypothetical protein ycgF	-1.101	0.019561
ydiL	hypothetical protein	-1.094	0.049093
ybhD	predicted DNA-binding transcriptional regulator	-1.090	0.011749
mhpE	4-hydroxy-2-ketovalerate aldolase	-1.081	0.041483
yjfM	hypothetical protein	-1.070	0.014448
cbl	DNA-binding transcriptional activator of cysteine biosynthesis	-1.069	0.042550
yddV	predicted diguanylate cyclase	-1.067	0.016753
cysC	adenylylsulfate kinase	-1.065	0.049261
c2220	Hypothetical protein	-1.064	0.046919
dhaR	predicted DNA-binding transcriptional regulator, dihydroxyacetone	-1.064	0.040124
c1185	Hypothetical protein	-1.062	0.030081
eutQ	hypothetical protein	-1.058	0.032359
yciW	predicted oxidoreductase	-1.055	0.031997
c2070	Hypothetical protein	-1.050	0.025263
gadX	DNA-binding transcriptional dual regulator	-1.042	0.009075
yjiN	Hypothetical protein yjiN	-1.039	0.013456
ydeJ	competence damage-inducible protein A	-1.029	0.031857
uxaA	altronate hydrolase	-1.027	0.030313
yjiL	predicted ATPase, activator of R)-hydroxyglutaryl-CoA dehydratase	-1.025	0.016041
ydhK	conserved inner membrane protein	-1.021	0.002014
yhiW	Hypothetical transcriptional regulator yhiW	-1.010	0.010194
dcuS	sensory histidine kinase in two-component regulatory system with DcuR, regulator of anaerobic fumarate respiration	-1.008	0.033020
wcaL	predicted glycosyl transferase	-1.000	0.008156

Table 24: ECC-1470 up-regulated genes at 3 h cocultivation with MAC-T cells

Gene	Description	Ratio	*p*-value
L7057	replication protein	5.790	0.002231
hypF	carbamoyl phosphate phosphatase and maturation protein for [NiFe] hydrogenases	5.457	0.000136
bglF	enzyme; Transport of small molecules: Carbohydrates, organic acids, alcohols	4.821	0.000549
hycG	hydrogenase 3 and formate hydrogenase complex, HycG subunit	4.739	0.001432
c3719	Hypothetical protein	4.516	0.000504
metR	Transcriptional activator protein metR	4.483	0.000550
gsp	bifunctional glutathionylspermidine amidase/glutathionylspermidine synthetase	4.306	0.000579
tauB	transport; Transport of small molecules: Amino acids, amines	3.965	0.000699
ygjO	predicted methyltransferase small domain	3.727	0.002154
ygjD	Probable O-sialoglycoprotein endopeptidase	3.673	0.002181
c1620	Hypothetical protein	3.656	0.000051
yiaY	predicted Fe-containing alcohol dehydrogenase	3.582	0.000580
oxyR	DNA-binding transcriptional dual regulator	3.537	0.001237
molR	-	3.535	0.001965
nfrA	bacteriophage N4 receptor, outer membrane subunit	3.530	0.000549
c4303	Putative conserved protein	3.462	0.000503
yfaZ	Hypothetical protein yfaZ precursor	3.455	0.001876

Gene	Description	Ratio	*p*-value
yjgB	predicted alcohol dehydrogenase, Zn-dependent and NAD(P)-binding	3.233	0.002593
c1956	Putative outer membrane protein yieC precursor	3.090	0.000090
hycD	hydrogenase 3, membrane subunit	2.984	0.000592
ppdA	hypothetical protein	2.964	0.000619
c4942	Hypothetical protein	2.942	0.000048
c3549	-	2.914	0.000527
ybhI	predicted transporter	2.909	0.005099
hydN	formate dehydrogenase-H, [4Fe-4S] ferredoxin subunit	2.896	0.007841
c3902	Hypothetical protein	2.869	0.000158
yehV	MerR-like regulator A	2.865	0.025096
ECP_3840	putative transposase	2.837	0.001371
uidC	predicted outer membrane porin protein	2.835	0.002436
astC	succinylornithine transaminase, PLP-dependent	2.792	0.000176
tauA	transport; Transport of small molecules: Amino acids, amines	2.780	0.002972
Z3309	putative tail fiber protein of prophage CP-933U	2.692	0.000359
c1584	putative tail component of prophage	2.637	0.007049
ybaT	predicted transporter	2.605	0.006390
menD	enzyme; Biosynthesis of cofactors, carriers: Menaquinone, ubiquinone	2.555	0.001016
ECs3006	putative C4-type zinc finger protein	2.547	0.000432
c3873	Putative conserved protein	2.542	0.006465
caiB	crotonobetainyl-CoA:carnitineCoA-transferase	2.539	0.001708
c0703	Hypothetical protein	2.506	0.001508
holE	DNA polymerase III, theta subunit	2.471	0.001682
ycdH	predicted oxidoreductase, flavin:NADH component	2.421	0.000258
yliL	hypothetical protein	2.356	0.013033
c3354	Hypothetical protein	2.317	0.002785
c5192	Conserved hypothetical protein	2.314	0.002924
c4059	Hypothetical protein	2.303	0.001040
menD	2-succinyl-6-hydroxy-2, 4-cyclohexadiene-1-carboxylate synthase	2.299	0.001156
c0317	Conserved hypothetical protein	2.262	0.008413
c1463	Hypothetical protein	2.248	0.000672
yqhD	alcohol dehydrogenase, NAD(P)-dependent	2.216	0.000985
yfaT	hypothetical protein	2.185	0.003653
mdtG	predicted drug efflux system	2.147	0.010446
ycdC	predicted DNA-binding transcriptional regulator	2.145	0.003293
ompN	outer membrane pore protein N, non-specific	2.112	0.010365
c0463	Hypothetical protein	2.092	0.010073
UTI89_C5126	putative tail component of prophage CP-933K	2.084	0.001359
yiaO	Putative ABC transporter periplasmic binding protein yiaO precursor	2.082	0.010065
c1589	putative tail component of prophage	2.060	0.007499
ybbW	predicted allantoin transporter	2.042	0.015666
yraQ	predicted permease	2.027	0.003642
hycH	protein required for maturation of hydrogenase 3	2.009	0.000028
ydcU	predicted spermidine/putrescine transporter subunit	1.999	0.007402
c4556	Conserved hypothetical protein	1.968	0.005309
nuoM	NADH dehydrogenase subunit M	1.941	0.006237
L7078	hypothetical protein	1.915	0.021250
ymgE	predicted inner membrane protein	1.884	0.000866
c3166	putative head-tail joining protein of prophage	1.871	0.004055
wcaB	Putative colanic acid biosynthesis acetyltransferase wcaB	1.866	0.001338
baeS	sensory histidine kinase in two-component regulatory system with BaeR	1.854	0.004573
yjbO	phage shock protein G	1.801	0.002748
cadB	predicted lysine/cadaverine transporter	1.796	0.047206
ypdI	predicted lipoprotein involved in colanic acid biosynthesis	1.790	0.007311
c2440	Hypothetical protein	1.774	0.005553
flhB	flagellar biosynthesis protein B	1.771	0.001443
cpsB	mannose-1-phosphate guanyltransferase	1.762	0.009126
mlrA	DNA-binding transcriptional regulator	1.762	0.006350
lldD	L-lactate dehydrogenase, FMN-linked	1.761	0.002179
srmB	ATP-dependent RNA helicase	1.743	0.000091
ybjJ	predicted transporter	1.743	0.006605
c1036	Hypothetical protein	1.742	0.002821
yjfC	predicted synthetase/amidase	1.729	0.004470

Gene	Description	Ratio	*p*-value
hyfI	hydrogenase 4, Fe-S subunit	1.718	0.000613
torC	enzyme; Energy metabolism, carbon: Anaerobic respiration	1.716	0.009041
yihQ	alpha-glucosidase	1.711	0.006721
klcA	KlcA	1.705	0.024550
insF	IS3 element protein InsF	1.692	0.009986
ycfA	hypothetical protein	1.686	0.000897
c0467	Hypothetical protein yaiO	1.668	0.002921
fdhF	formate dehydrogenase-H, selenopolypeptide subunit	1.668	0.010215
hycF	formate hydrogenlyase complex iron-sulfur protein	1.642	0.018023
sgbH	3-keto-L-gulonate 6-phosphate decarboxylase	1.637	0.016103
yjaB	predicted acetyltransferase	1.607	0.009422
mdtI	multidrug efflux system transporter	1.588	0.008813
arcA	DNA-binding response regulator in two-component regulatory system with ArcB or CpxA	1.581	0.000107
yegS	hypothetical protein	1.580	0.005754
yhhW	hypothetical protein	1.559	0.008664
fadA	3-ketoacyl-CoA thiolase	1.546	0.015743
hyaA	hydrogenase 1, small subunit	1.542	0.007352
nhoA	N-hydroxyarylamine O-acetyltransferase	1.539	0.009754
insF	IS3 element protein InsF	1.530	0.001892
hycC	NADH dehydrogenase subunit N	1.522	0.003785
potH	putrescine transporter subunit: membrane component of ABC superfamily	1.522	0.015898
insF	IS3 element protein InsF	1.515	0.007252
mglB	methyl-galactoside transporter subunit	1.501	0.006562
yhcM	conserved protein with nucleoside triphosphate hydrolase domain	1.500	0.010056
hyfF	NADH dehydrogenase subunit N	1.499	0.016042
yehD	Hypothetical protein yehD precursor	1.492	0.017652
pflB	pyruvate formate lyase I	1.491	0.005179
c2092	Hypothetical protein	1.490	0.026718
yidL	predicted DNA-binding transcriptional regulator	1.482	0.011844
c5302	Hypothetical protein	1.478	0.005414
rbn	ribonuclease BN	1.478	0.012602
Z0980	putative tail component of prophage CP-933K	1.467	0.012893
rbsR	DNA-binding transcriptional repressor of ribose metabolism	1.452	0.013740
insF	IS3 element protein InsF	1.445	0.010733
ypfE	predicted carboxysome structural protein with predicted role in ethanol utilization	1.444	0.012797
gadC	predicted glutamate:gamma-aminobutyric acid antiporter	1.429	0.004421
ygjQ	Hypothetical protein ygjQ	1.427	0.007707
glcD	glycolate oxidase subunit, FAD-linked	1.405	0.002116
c0784	Hypothetical protein	1.391	0.001195
ompW	Outer membrane protein W precursor	1.388	0.000607
yhhK	hypothetical protein	1.380	0.005911
hipA	regulator; Murein sacculus, peptidoglycan	1.378	0.027696
insF	IS3 element protein InsF	1.376	0.006192
ygfQ	predicted transporter	1.367	0.010043
malY	bifunctional beta-cystathionase, PLP-dependent/ regulator of maltose regulon	1.363	0.001670
kdpD	fused sensory histidine kinase in two-component regulatory system with KdpE: signal sensing protein	1.358	0.000920
hycE	hydrogenase 3, large subunit	1.353	0.001815
crp	DNA-binding transcriptional dual regulator	1.352	0.000107
ydiY	hypothetical protein	1.348	0.004095
gadB	glutamate decarboxylase B, PLP-dependent	1.336	0.002298
glnP	glutamine ABC transporter permease protein	1.333	0.024318
ddpC	D-ala-D-ala transporter subunit	1.332	0.022925
Z3082	putative tail fiber component L of prophage CP-933U	1.332	0.010098
yfeW	hypothetical protein	1.322	0.006524
nhoA	N-hydroxyarylamine O-acetyltransferase	1.317	0.008270
cusS	sensory histidine kinase in two-component regulatory system with CusR, senses copper ions	1.308	0.033305
napA	nitrate reductase, periplasmic, large subunit	1.308	0.011477
menE	O-succinylbenzoic acid--CoA ligase	1.304	0.016058
yjiH	Hypothetical protein yjiH	1.297	0.017380

Gene	Description	Ratio	*p*-value
metE	5-methyltetrahydropteroyltriglutamate-- homocysteine methyltransferase	1.295	0.004490
ybhM	conserved inner membrane protein	1.270	0.017594
ECP_2970	fimbrial usher protein PixC	1.269	0.001881
ypdI	Hypothetical lipoprotein ypdI precursor	1.266	0.001286
bcsE	hypothetical protein	1.262	0.008006
mdtL	multidrug efflux system protein	1.255	0.009388
citE	enzyme; Central intermediary metabolism: Pool, multipurpose conversions	1.254	0.014851
ycjB	hypothetical protein	1.254	0.005623
gamW	putative factor; Integration, recombination (Phage or Prophage Related)	1.247	0.008788
ygeD	predicted inner membrane protein	1.247	0.001942
Z3143	hypothetical protein	1.240	0.015019
yihM	Hypothetical protein yihM	1.238	0.025905
nikE	ATP-binding protein of nickel transport system	1.234	0.010987
malG	maltose transporter subunit	1.233	0.012644
ulaA	ascorbate-specific PTS system enzyme IIC	1.224	0.012695
Z4186	putative integral membrane protein-component of typeIII secretion apparatus	1.216	0.005120
c3663	Hypothetical protein	1.215	0.016870
gltD	glutamate synthase, 4Fe-4S protein, small subunit	1.215	0.025390
deoR	DNA-binding transcriptional repressor	1.209	0.001210
sbmC	DNA gyrase inhibitor	1.208	0.006187
yfeU	Protein yfeU	1.207	0.008644
gatB	galactitol-specific enzyme IIB component of PTS	1.205	0.012821
c4837	Hypothetical protein	1.203	0.003203
umuC	DNA polymerase V subunit UmuC	1.200	0.016061
ECs1173	hypothetical protein	1.199	0.004441
tauB	taurine transporter subunit	1.197	0.003452
ccmD	cytochrome c biogenesis protein	1.196	0.007642
ilvC	enzyme; Amino acid biosynthesis: Isoleucine, Valine	1.195	0.022177
ivbL	ilvB operon leader peptide	1.192	0.003412
c3164	putative factor; DNA packaging, phage assembly (Phage or Prophage Related)	1.186	0.005155
nikB	nickel transporter subunit	1.179	0.013204
argD	bifunctional acetylornithine aminotransferase/ succinyldiaminopimelate aminotransferase	1.175	0.035302
argB	Acetylglutamate kinase	1.164	0.009083
hdeD	HdeD protein	1.164	0.003032
rluA	pseudouridine synthase for 23S rRNA (position 746) and tRNAphe(position 32)	1.146	0.005095
yeiM	predicted nucleoside transporter	1.144	0.029193
astB	succinylarginine dihydrolase	1.142	0.015883
yabI	conserved inner membrane protein	1.135	0.024346
yjgH	predicted mRNA endoribonuclease	1.135	0.021796
hisD	Histidinol dehydrogenase	1.129	0.002684
yjbG	hypothetical protein	1.126	0.009933
-	positive control stringency: 90% identity to oligo b2965	1.124	0.002593
yohL	hypothetical protein	1.122	0.004686
cysU	sulfate/thiosulfate transporter subunit	1.120	0.019483
yghD	Putative general secretion pathway protein M-type yghD	1.120	0.018123
galE	UDP-galactose-4-epimerase	1.116	0.002901
ymgG	hypothetical protein	1.112	0.009072
UTI89_C1520	hypothetical protein	1.110	0.001982
yhdX	predicted amino-acid transporter subunit	1.108	0.003997
ybjH	hypothetical protein	1.104	0.027399
c4868	Hypothetical protein	1.102	0.003302
cysE	Serine acetyltransferase	1.097	0.004214
dppC	dipeptide transporter	1.096	0.019518
ubiF	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	1.096	0.014956
hyfG	hydrogenase 4, subunit	1.094	0.003590
yeaJ	predicted diguanylate cyclase	1.085	0.005876
ycjX	conserved protein with nucleoside triphosphate hydrolase domain	1.083	0.011332
yedF	hypothetical protein	1.079	0.008902
UTI89_C3197	ClpB protein	1.075	0.001114
ECs5296	hypothetical protein	1.074	0.018692
torY	TMAO reductase III (TorYZ), cytochrome c-type subunit	1.074	0.012790
htrG	predicted signal transduction protein (SH3 domain)	1.073	0.003127

Gene	Description	Ratio	*p*-value
speC	ornithine decarboxylase, constitutive	1.073	0.008918
ycaI	conserved inner membrane protein	1.073	0.002607
ccmF	-	1.071	0.047346
hisF	imidazole glycerol phosphate synthase subunit HisF	1.059	0.019141
ybbI	putative transcriptional regulator	1.050	0.022351
rhaT	L-rhamnose:proton symporter	1.048	0.012595
c0315	Hypothetical protein	1.047	0.001187
rhsA_B	rhsA element core protein RshA | rhsB element core protein RshB	1.047	0.012295
yjiS	hypothetical protein	1.047	0.021365
hycA	regulator of the transcriptional regulator FhlA	1.046	0.023478
UTI89_C2967	bacteriophage V tail protein	1.034	0.004356
yhaR	hypothetical protein	1.032	0.016815
narV	nitrate reductase 2 (NRZ), gamma subunit	1.030	0.022004
hdeB	protein hdeB precursor (10K-L protein)	1.029	0.029747
menC	enzyme; Biosynthesis of cofactors, carriers: Menaquinone, ubiquinone	1.028	0.021598
msrA	methionine sulfoxide reductase A	1.027	0.011569
ygfU	Putative purine permease ygfU	1.027	0.005518
hisF	imidazole glycerol phosphate synthase subunit HisF	1.026	0.000399
c2748	Hypothetical protein	1.023	0.009643
c3404	Hypothetical protein	1.023	0.020023
fadH	2,4-dienoyl-CoA reductase, NADH and FMN-linked	1.019	0.011222
lsrC	AI2 transporter	1.018	0.009215
flhE	hypothetical protein	1.012	0.010775
gadA	glutamate decarboxylase A, PLP-dependent	1.012	0.032270
c5221	Hypothetical protein	1.010	0.003017
phnI	carbon-phosphorus lyase complex subunit	1.010	0.019953
yegH	fused predicted membrane protein/predicted membrane protein	1.009	0.014991
Z0961	putative endopeptidase protein Rz of prophage CP-933K	1.008	0.013013
yhhX	predicted oxidoreductase with NAD(P)-binding Rossmann-fold domain	1.007	0.002372

Table 25: ECC-1470 down-regulated genes at 3 h cocultivation with MAC-T cells

Gene	Description	Ratio	*p*-value
ybiM	Hypothetical protein ybiM	-4.300	0.012010
yjgG_2	hypothetical protein	-4.082	0.009680
citD	citrate lyase, acyl carrier (gamma) subunit	-4.070	0.013448
Z3104	putative endolysin of prophage CP-933U	-4.034	0.009911
araD	L-ribulose-5-phosphate 4-epimerase	-3.976	0.007142
tnaC	tryptophanase leader peptide	-3.824	0.025220
yccE	hypothetical protein	-3.643	0.003228
yibJ	predicted Rhs-family protein	-3.626	0.006475
htrE	predicted outer membrane usher protein	-3.227	0.030739
yahF	predicted acyl-CoA synthetase with NAD(P)-binding domain and succinyl-CoA synthetase domain	-3.063	0.016091
cusC	copper/silver efflux system, outer membrane component	-2.928	0.017598
phnH	carbon-phosphorus lyase complex subunit	-2.848	0.027044
ycgX	hypothetical protein	-2.807	0.026261
ybeQ	hypothetical protein	-2.785	0.036663
yadD	predicted transposase	-2.609	0.006534
ycaM	predicted transporter	-2.601	0.010163
tfaQ	Qin prophage; predicted tail fibre assembly protein	-2.445	0.004050
tdcE	pyruvate formate-lyase 4/2-ketobutyrate formate-lyase	-2.326	0.049898
dgoD	galactonate dehydratase	-2.245	0.001587
lsrA	fused AI2 transporter subunits of ABC superfamily: ATP-binding components	-2.218	0.001154
ybbD	hypothetical protein	-2.192	0.026042
csgC	predicted curli production protein	-2.163	0.015114
lamB	maltoporin precursor	-2.037	0.039822
ydiF	fused predicted acetyl-CoA:acetoacetyl-CoA	-1.990	0.006219
Z2118	putative endopeptidase Rz of prophage CP-933O	-1.898	0.009605
yncK	-	-1.871	0.029613
ybcM	DLP12 prophage; predicted DNA-binding transcriptional regulator	-1.870	0.014808

Gene	Description	Ratio	*p*-value
c3104	Hypothetical protein	-1.846	0.034249
dmsC	dimethyl sulfoxide reductase, anaerobic, subunit C	-1.842	0.004755
molR	-	-1.836	0.001920
rbsA	fused D-ribose transporter subunits of ABC superfamily: ATP-binding components	-1.809	0.045647
ydhV	predicted oxidoreductase	-1.743	0.038687
ykgK	predicted regulator	-1.739	0.013657
hofB	conserved protein with nucleoside triphosphate hydrolase domain	-1.725	0.009491
Z1879	putative envelope protein of prophage CP-933X	-1.695	0.002700
ydhW	hypothetical protein	-1.688	0.037369
ydhK	conserved inner membrane protein	-1.688	0.012888
argI	ornithine carbamoyltransferase 1	-1.647	0.015689
yqeJ	hypothetical protein	-1.621	0.024657
phnE	phosphonate/organophosphate ester transporter pseudogene)	-1.620	0.029698
c1893	Hypothetical protein	-1.620	0.004923
guaD	guanine deaminase	-1.604	0.021913
alx	predicted inner membrane protein, part of terminus	-1.593	0.017622
ybbS	DNA-binding transcriptional activator of the allD operon	-1.583	0.017958
fdnH	formate dehydrogenase-N, Fe-S (beta) subunit, nitrate-inducible	-1.571	0.040879
yeaT	predicted DNA-binding transcriptional regulator	-1.562	0.012233
yqeA	predicted amino acid kinase	-1.557	0.023517
ydfO	Qin prophage; predicted protein	-1.552	0.007130
ybcF	predicted carbamate kinase	-1.525	0.040903
ECs4865	hypothetical protein	-1.510	0.030855
ycaC	predicted hydrolase	-1.486	0.000411
c1465	putative factor; DNA packaging, phage assembly (Phage or Prophage Related)	-1.471	0.001336
htrC	heat shock protein	-1.468	0.003033
ycaI	hypothetical protein	-1.452	0.020957
cynT	carbonic anhydrase	-1.443	0.030744
dgoA	2-dehydro-3-deoxy-6-phosphogalactonate aldolase	-1.441	0.024391
aaeA	p-hydroxybenzoic acid efflux system component	-1.441	0.008529
Z2145	putative tail component of prophage CP-933O	-1.428	0.006892
uidR	DNA-binding transcriptional repressor	-1.428	0.033644
ybhD	predicted DNA-binding transcriptional regulator	-1.421	0.017823
ydhY	predicted 4Fe-4S ferridoxin-type protein	-1.414	0.006821
bglH	carbohydrate-specific outer membrane porin, cryptic	-1.402	0.038043
potE	putrescine/proton symporter:	-1.400	0.034283
mtlR	DNA-binding repressor	-1.374	0.029068
ymgB	hypothetical protein	-1.366	0.041345
ybfC	hypothetical protein	-1.366	0.004805
eutG	predicted alcohol dehydrogenase in ethanolamine utilization	-1.349	0.004543
ydiP	predicted DNA-binding transcriptional regulator	-1.338	0.029553
chbF	cryptic phospho-beta-glucosidase, NAD(P)-binding	-1.337	0.000827
sbp	sulfate transporter subunit	-1.336	0.002818
ytfA	predicted transcriptional regulator	-1.333	0.019656
dmsB	dimethyl sulfoxide reductase, anaerobic, subunit B	-1.314	0.002435
yiaW	hypothetical protein	-1.300	0.012544
lysA	diaminopimelate decarboxylase, PLP-binding	-1.275	0.018981
fumC	fumarate hydratase	-1.267	0.047930
ybeF	predicted DNA-binding transcriptional regulator	-1.256	0.012955
ydcP	predicted peptidase	-1.254	0.015597
ybfL	predicted transposase (pseudogene)	-1.238	0.029371
c2235	Conserved hypothetical protein	-1.222	0.013779
c1936	-	-1.220	0.048299
c1138	Hypothetical protein	-1.214	0.031859
bglG	transcriptional antiterminator of the bgl operon	-1.210	0.033472
glvG	predicted 6-phospho-beta-glucosidase pseudogene)	-1.204	0.010783
aegA	fused predicted oxidoreductase: FeS binding	-1.200	0.001871
c3702	Hypothetical protein	-1.188	0.027166
ycaL	predicted peptidase with chaperone function	-1.187	0.008718
ylbH	conserved protein, rhs-like	-1.182	0.017094
ECs2713	hypothetical protein	-1.171	0.014653
yhaR	TdcF protein	-1.167	0.015581

Gene	Description	Ratio	*p*-value
glpT	sn-glycerol-3-phosphate transporter	-1.160	0.027285
Z0722	hypothetical protein	-1.156	0.007267
glcC	DNA-binding transcriptional dual regulator, glycolate-binding	-1.152	0.008660
hyaD	protein involved in processing of HyaA and HyaB proteins	-1.146	0.000404
pheM	phenylalanyl-tRNA synthetase operon leader peptide	-1.144	0.018139
c0690	Hypothetical protein ybdN	-1.137	0.043568
ybcL	DLP12 prophage; predicted kinase inhibitor	-1.136	0.040394
prpR	DNA-binding transcriptional activator	-1.136	0.031221
cpxP	periplasmic protein combats stress	-1.132	0.009376
dos	cAMP phosphodiesterase, heme-regulated	-1.130	0.014659
ybhJ	Hypothetical protein ybhJ	-1.129	0.003394
sprT	hypothetical protein	-1.129	0.000123
yfaZ	predicted outer membrane porin protein	-1.127	0.049902
ycjD	hypothetical protein	-1.123	0.030185
edd	phosphogluconate dehydratase	-1.115	0.016328
yhhI	predicted transposase	-1.110	0.018119
Z2208	hypothetical protein	-1.105	0.040136
yfgH	predicted outer membrane lipoprotein	-1.104	0.017593
astA	arginine succinyltransferase	-1.104	0.009231
rus	endodeoxyribonuclease RUS (Holliday junction resolvase) of prophage CP-933X	-1.101	0.017612
creD	inner membrane protein	-1.100	0.005758
yadL	predicted fimbrial-like adhesin protein	-1.097	0.001078
yobD	Hypothetical protein yobD	-1.091	0.012583
ibpB	heat shock chaperone	-1.085	0.028868
Z5924	hypothetical protein	-1.084	0.003007
rusA	DLP12 prophage; endonuclease RUS	-1.083	0.022880
melA	alpha-galactosidase, NAD(P)-binding	-1.078	0.003420
yciW	predicted oxidoreductase	-1.072	0.006564
ylcE	DLP12 prophage; predicted protein	-1.069	0.001720
recE	Exodeoxyribonuclease VIII	-1.069	0.032374
c5065	Hypothetical protein	-1.067	0.031807
c2318	Hypothetical protein	-1.065	0.007308
ECs2770	hypothetical protein	-1.058	0.021718
yidP	predicted DNA-binding transcriptional regulator	-1.046	0.021587
pflD	predicted formate acetyltransferase 2 (pyruvate formate lyase II)	-1.046	0.032995
paaJ	acetyl-CoA acetyltransferase	-1.043	0.031153
ybiU	hypothetical protein	-1.037	0.016855
yjgI	predicted oxidoreductase with NAD(P)-binding Rossmann-fold domain	-1.034	0.019497
ytfE	predicted regulator of cell morphogenesis and cell wall metabolism	-1.032	0.041299
ybiA	hypothetical protein	-1.029	0.017831
ybdM	hypothetical protein	-1.025	0.003990
Z0964	putative DNA packaging protein of prophage CP-933K	-1.016	0.030727
thiH	thiamine biosynthesis protein ThiH	-1.005	0.000352
ccmB	heme exporter subunit	-1.005	0.018704
yieK	hypothetical protein	-1.002	0.010502

VIII.2.2. Transcriptome data of RNA-Seq

Data derived from differential gene expression profiling of mastitis *E. coli* 1303 and ECC-1470 cultivated in DMEM, milk whey and LPS challenged milk whey, respectively, sorted according to their level of expression. Data are presented on compact disk:

Supplemental Tables S2 – S7: Expression values of the individual samples from RNA-Seq

Supplemental Tables S8 – S10: Differential expression of genes from RNA-Seq by bROC algorithm

VIII.3. Curriculum Vitae

Personal Data

Name: Ingmar Erik Zude

Address: Frühlingstr. 45, 22525 Hamburg

Date of birth: October 28th, 1981

Place of birth: Hamburg, Germany

Education

2008-2014 PhD thesis in behalf of the International Graduate School of Würzburg
as a member of the Graduate School of Life Sciences
at the University of Würzburg in the Inst. of Molecular Infection Biology in 2008-2010
at the University Hospital of Münster in the Inst. of Hygiene in 2010-2013

2002-2008 Diploma in Biology at the University of Würzburg, Germany

Main subjects: Molecular Microbiology and Infectiology
Ecophysiology of Plants
Molecular Physiology of Plants
Pharmaceutical Biology

Diploma Thesis: Functional characterization of autotransporters in *E. coli* strain 536

1992-2001 Gymnasium Dörpsweg in Hamburg, Germany

Main subjects: Biology, Chemistry, German and Philosophy,

Hamburg, Mai 2014 ……

VIII.4. Publications

Publication

Zude, I., Leimbach, A., and Dobrindt, U. (2013) Prevalence of autotransporters in *Escherichia coli*: what is the impact of phylogeny and pathotype? *Int J Med Microbiol* ahead of print (DOI: 10.1016/j.ijmm.2013.10.006)

Presentations

Zude, I., Hacker, J. and Dobrindt, U., (2009) Functional characterization of autotransporters in *E. coli.* Students of the GSLS Würzburg, 4th International Symposium - Revolution Research, Würzburg, Poster presentation

Zude, I., Hacker, J. and Dobrindt, U., (2009) Functional characterization of autotransporters in *E. coli.* SFB 479 International Symposium, Living with pathogens – never lose control, Würzburg, Poster presentation

Zude, I., (2009) Functional characterization of autotransporters in *E. coli.* Forschungszentrum Borstel – Leibniz-Zentrum für Medizin und Biowissenschaften, FOR585 Meeting, Borstel, Oral presentation

Zude, I., (2010) Characterization of virulence-associated traits of *Escherichia coli* bovine mastitis isolates. Joint conference: 43rd Annual conference, Physiology und Pathology of Reproduction, 34th Veterinary and human medicine joint conference, Munich, Oral presentation

Zude, I., Plaschke, B., Reichardt, E., Köhler, C.-D., Petzl, W., Zerbe, H., Seyfert, H.-M., and Dobrindt, U. (2010) Characterization of virulence-associated traits of *Escherichia coli* bovine mastitis isolates. 3rd Joint Conference, German Society for Hygiene and Microbiology (DGHM), Association for General and Applied Microbiology (VAAM), Hannover, Poster presentation

Zude, I., Plaschke, B., Reichardt, E., Köhler, C.-D., Petzl, W., Zerbe, H., Seyfert, H.-M., and Dobrindt, U. (2010) Characterization of virulence-associated traits of *Escherichia coli* bovine mastitis isolates. Network of Excellence, EuroPathoGenomics Graduate Academy, "European Virtual Institute for Functional Genomics of Bacterial Pathogens", Pécs, Poster presentation

Zude, I., (2010) Characterization of virulence-associated traits of *Escherichia coli* bovine mastitis isolates. Institute for Molecular Infection Biology, FOR585 Meeting, Würzburg, Oral presentation

Zude, I., Plaschke, B., Reichardt, E., Köhler, C.-D., Petzl, W., Zerbe, H., Seyfert, H.-M., and Dobrindt, U. (2010) Characterization of virulence-associated traits of *Escherichia coli* bovine mastitis isolates. Students of the GSLS Würzburg, 5th international Symposium – CHIASMA on the crossroads of research, Würzburg, Poster presentation

Zude, I., Petzl, W., Zerbe, H., Seyfert, H.-M., and Dobrindt, U. (2011) Characterization of virulence-associated traits of *Escherichia coli* bovine mastitis isolates. Leibniz Institute for Farm Animal Biology, International Conference on the Physiology and Genomics of Mastitis, Tutzing, Poster presentation

VIII.5. Abbreviations

µg microgram
µl microliter
µM micromolar
µm micrometer
A adenine
Amp ampicillin
bp base pairs
BSA bovine serum albumin
C cytosine
CFU colony forming unit
DE differential expression
DEPC diethyl pyrocarbinate
DNA deoxyribonucleic acid
DNase deoxyribonuclease
EAEC enteroaggregative *E. coli*
Ec Escherichia coli
EDTA ethylendiamintertraacetat
e.g. *exempli gratia* (for example)
EHEC enterohemorrhagic *E. coli*
EIEC enteroinvasive *E. coli*
EPEC enteropathogenic *E. coli*
et al. *et altera* (and others)
ETEC enterotoxigenic *E. coli*
EtOH ethanol
ExPEC extraintestinal pathogenic *E. coli*
g gram
G guanine
GEI genomic island
h hour
IL Interleukin
IPEC intestinal pathogenic *E. coli*
kb kilo bases
Km kanamycin
l liter
LB lysogeny broth
LPS lipopolysaccharide acid
mg milligram
min minute
ml milliliter
mm millimeter
mM millimolar
ng nanogramm
NO nitric oxide
nt nucleotides
OD optical density
ORF open reading frame
PAI pathogenicity island
PBS phosphate buffered saline
PCR polymerase chain reaction
RNA ribonucleic acid
RNase ribonuclease
rpm rounds per minute
RT reverse transcription
RT-PCR reverse transcription PCR
SD standard deviation
SDS sodium dodecyl sulfate
sec second
SEM standard error of the mean
SNP single nucleotide polymorphism
ß-ME beta-mercaptoethanol
T thymine
TAE Tris-acetate-EDTA
TLR4 toll like receptor 4
UPEC uropathogenic *E. coli*
UTI urinary tract infection
wt wild type