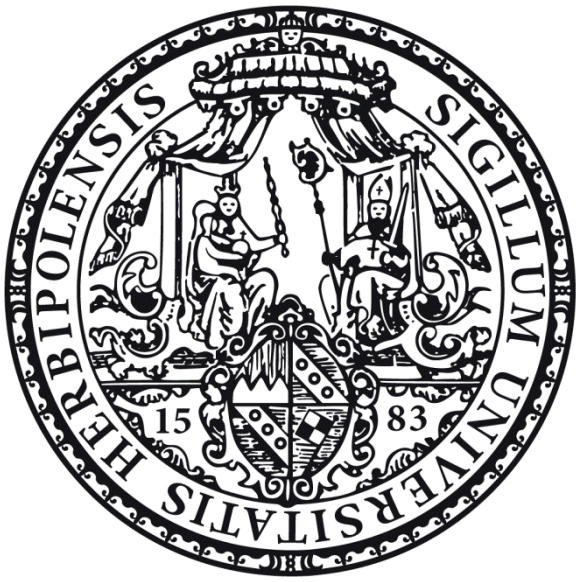

Morphology, regulation and interstrain interactions in a new macrocolony biofilm model of the human pathogen *Staphylococcus aureus*

Morphologie, Regulation und stammübergreifende Wechselwirkungen in einem neuen Makrokolonie-Biofilmmodell des Humanpathogens *Staphylococcus aureus*

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Summary

The role of multicellularity as the predominant microbial lifestyle has been affirmed by studies on the genetic regulation of biofilms and the conditions driving their formation. Biofilms are of prime importance for the pathology of chronic infections of the opportunistic human pathogen *Staphylococcus aureus*.

The recent development of a macrocolony biofilm model in *S. aureus* opened new opportunities to study evolution and physiological specialization in biofilm communities in this organism. In the macrocolony biofilm model, bacteria form complex aggregates with a sophisticated spatial organization on the micro- and macroscale. The central positive and negative regulators of this organization in *S. aureus* are the alternative sigma factor σ^B and the quorum sensing system Agr, respectively. Nevertheless, nothing is known on additional factors controlling the macrocolony morphogenesis.

In this work, the genome of *S. aureus* was screened for novel factors that are required for the development of the macrocolony architecture. A central role for basic metabolic pathways was demonstrated in this context as the macrocolony architecture was strongly altered by the disruption of nucleotide and carbohydrate synthesis. Environmental signals further modulate macrocolony morphogenesis as illustrated by the role of an oxygen-sensitive gene regulator, which is required for the formation of complex surface structures. A further application of the macrocolony biofilm model was demonstrated in the study of interstrain interactions. The integrity of macrocolony communities was macroscopically visibly disturbed by competitive interactions between clinical isolates of *S. aureus*.

The results of this work contribute to the characterization of the macrocolony biofilm model and improve our understanding of developmental processes relevant in staphylococcal infections. The identification of anti-biofilm effects exercised through competitive interactions could lead to the design of novel antimicrobial strategies targeting multicellular bacterial communities.

Zusammenfassung

Die Rolle von Multizellularität als der vorherrschende mikrobielle Lebensstil wurde durch Studien über die genetische Steuerung von Biofilmen und über Biofilmbildung-fördernde Bedingungen bestätigt. Biofilme sind wichtige Faktoren in der Pathogenese chronischer Infektionen durch das opportunistische Humanpathogen *Staphylococcus aureus*.

Die kürzlich erfolgte Entwicklung eines Makrokolonie-Biofilmmodells für *S. aureus* eröffnet neue Möglichkeiten evolutionäre Entwicklungen und die physiologische Spezialisierung in bakteriellen Gemeinschaften zu untersuchen. Im Makrokolonie-Biofilmmodell bilden Bakterien komplexe Aggregate, die sich durch eine hochentwickelte räumliche Organisation auf mikroskopischer und makroskopischer Ebene auszeichnen. Die positiven und negativen Hauptregulatoren dieser Organisation sind der alternative Sigmafaktor σ^B sowie das Quorum sensing System Agr. Dennoch sind weitere Faktoren, die die Morphogenese der Makrokolonien steuern, unbekannt.

In dieser Arbeit wurde das Genom von *S. aureus* im Hinblick auf neue Faktoren, die für die Entwicklung der Makrokoloniearchitektur nötig sind, analysiert. Dabei wurde belegt, dass zentrale Stoffwechselwege eine zentrale Rolle spielen. Störungen der Nukleotid- und Kohlenhydrat-Synthese hatten starke Auswirkungen auf die Makrokoloniearchitektur. Weiterhin wurde anhand eines Sauerstoff-sensitiven Genregulators, der für die Ausbildung von Oberflächenstrukturen nötig ist, demonstriert, wie die Morphogenese der Makrokolonien durch Umweltsignale moduliert wird. Das Makrokolonie-Biofilmmodell fand weitere Anwendung in der Untersuchung von stammübergreifenden Interaktionen. Die Integrität der Makrokolonie-Biofilme wurde durch die Wechselwirkungen in Konkurrenz stehender klinischer Isolate stark herabgesetzt.

Die Ergebnisse dieser Arbeit tragen zur Charakterisierung des Makrokolonie-Biofilmmodells bei und geben Einsicht in Entwicklungsprozesse, die während Staphylokokken-Infektionen ablaufen. Die Beschreibung der negativen Beeinflussung der Biofilme durch bakterielle

Wechselwirkungen könnte zur Entwicklung neuer antimikrobieller Strategien, die gezielt gegen multizelluläre bakterielle Gemeinschaften wirksam sind, beitragen.

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Preface

Bacterial pathogens, which are a leading cause of death and create significant morbidity, have long been a burden on society (Fonkwo, 2008, Lozano et al., 2012). The discovery of antimicrobial substances has greatly improved treatment options and enabled medical progress in many fields (Nathan and Cars, 2014). Continued research on novel antimicrobial strategies is necessitated by the emergence of antibiotic resistances and the increasing knowledge on drug-tolerant subpopulations.

The versatility of bacteria to adapt to changing environments makes it necessary to develop innovative model systems that orient themselves on the ecological niches during infections. The macrocolony biofilm model of *Staphylococcus aureus* is such a model as it mimics the conditions found in magnesium-rich host tissues (Garcia-Betancur et al., 2017, Koch et al., 2014b). In addition to research on biofilm-related processes (e.g. the organization of the community), microbial macrocolony models are suitable for studies regarding evolution, differentiation and adaptation to conditions in the host.

Two different projects related to developmental processes in macrocolony biofilms of *S*. *aureus* are addressed in this thesis. Firstly, the genetic requirements for the formation of macroscale structures in the macrocolony biofilm model are studied. Three cellular functions that are identified as new factors in the regulation of colony morphology are reviewed in section I.3 of the introduction. The second central aspect of this work pertains to the characterization of phage-mediated interstrain competition in biofilm communities. The sections I.4 and I.5 of the introduction focus on presenting fundamental principles of ecological interactions in biofilms and review the field of bacteriophage biology in *S*. *aureus*.

I. Introduction

I.1 *Staphylococcus aureus*

I.1.1 Clinical impact of *S. aureus*

It is estimated that 1 in 14 patients contracts a hospital associated infection (HAI) during their hospitalization in the European Union (EU) (ECDC, 2012). Overall, HAIs affect more than 4 million patients in the EU annually and create additional costs of more than 7 billion euro in the health care sector (ECDC, 2012). At least 60% of HAIs are associated with the formation of biofilms, either during the course of the infection or because the infection is caused by bacteria that disperse from a biofilm into the body (Klevens et al., 2007). The biofilm producer *Staphylococcus aureus* is among the most prevalent pathogens that are associated with HAIs (Dantes et al., 2011, Jarvis and Martone, 1992, Khan et al., 2015, Klein et al., 2007, Richards et al., 1999). *S. aureus* is frequently isolated from hospitalized patients with pneumonia, bloodstream infections and surgical site infections, especially in intensive care units (Magill et al., 2014). Not all *S. aureus* infections in hospitalized patients are nosocomial; they can also be primary community-acquired infections that require hospitalization (Kuehnert et al., 2005, Sampedro and Bubeck Wardenburg, 2017). The initial isolation of *S. aureus* was performed from an infected surgical wound, still a commonplace for staphylococcal infections more than a century later (Magill et al., 2014, Ogston, 1881). *S. aureus* was identified as a coccal bacterium growing in grape-like clusters with a characteristic golden color due to the pigmentation with the carotenoid staphyloxanthin (Marshall, 1972, Ogston, 1882).

S. aureus has developed resistance against most classes of clinically-used antibiotics, most notably methicillin but also against last-resort antibiotics, e.g. vancomycin (Chambers and Deleo, 2009). Penicillin was the first commercially available antibiotic in 1940 (Chain et al., 1940). Penicillin is a β-lactam antibiotic that acts on the penicillin-binding proteins (PBP) of the *S. aureus* cell wall. PBPs are transpeptidases that recognize the peptide side chains of the peptidoglycan building blocks and crosslink newly synthesized peptidoglycan with the

existing cell wall. B-lactams are structural analogues of the PBP target and weaken the cell wall by interfering with the crosslinking reaction (Waxman and Strominger, 1983).

Penicillin-resistant strains, which were isolated early after the beginning of widespread use of penicillin, overcome the bactericidal effect of the antibiotic by secretion of a β-lactamase that renders the substance inactive (Barber and Rozwadowska-Dowzenko, 1948, Kirby, 1944, Olsen et al., 2006, Rammelkamp and Maxon, 1942). The β-lactamase is encoded by the gene *blaZ* and closely linked to the repressor BlaR as well as the sensory protein BlaI (Hackbarth and Chambers, 1993, Olsen et al., 2006, Zhang et al., 2001). The β-lactamase producing unit can occur on mobile genetic elements like plasmids and transposons but also on the core chromosome of *S. aureus* (Malachowa and DeLeo, 2010, Olsen et al., 2006). Methicillin and the more stable derivative oxacillin were developed as β-lactamase-stable derivatives of penicillin (Rutenburg et al., 1960, Simon and Rantz, 1962). However, shortly after their introduction methicillin-resistant *S. aureus* (MRSA) strains were isolated from many hospitals worldwide which lead to the new differentiation between methicillin-sensitive *S. aureus* (MSSA) and MRSA strains (Barber, 1961, Jevons, 1961). The resistance against methicillin and its derivatives is not based on drug inactivation. Instead, resistance is conferred by the alternative penicillin-binding protein PBP2a (or PBP2') encoded by *mecA* (Matsuhashi et al., 1986). PBP2a has a lower affinity to β-lactams and remains functional in their presence (Hartman and Tomasz, 1984). Interestingly, *mecA* is found on a mobile genetic element – the staphylococcal chromosome cassette SCCmec – and can thus be transferred horizontally contributing to the rapid spread of MRSA strains (Ito et al., 2001, Katayama et al., 2000). Different types of SCCmec have been associated with waves of dominant MRSA lineages (Chambers and Deleo, 2009). Additionally to providing resistance against β-lactam antibiotics, SCCmec types II and III were found to contain resistance genes against non-β-lactams (Deurenberg et al., 2007). The currently dominant MRSA lineage (USA300) carries SCCmec IV, which contains no additional resistance genes (Diep et al., 2006, Glaser et al., 2016).

I.1.2 *S. aureus* pathogenesis

Despite its notoriety for antibiotic resistance and as a pathogen in intensive care units, interactions between *S. aureus* and humans are benign in most circumstances. While the incidence of invasive infections with *S. aureus* is around 34 cases / 100,000 population, around 30% of the population is permanently asymptomatically colonized with *S. aureus* in the nares (Becker et al., 2017, Jacobsson et al., 2007). The likeliness of a contact of the immune system with *S. aureus* has been calculated to be 1000 x higher in the nares compared to an invasive infection (Laupland et al., 2003). Nevertheless, nasal carriage was identified as the biggest reservoir for invasive infections and decolonization can reduce the risk to contract a HAI with *S. aureus* (Bode et al., 2010, von Eiff et al., 2001, Young et al., 2017, Yu et al., 1986).

Besides asymptomatic colonization of the nares, *S. aureus* can affect a large variety of tissues with different outcomes. Similar to the nares, colonization of the skin is often asymptomatic, but breaches of the skin can lead to superficial skin and soft tissue infections (Dryden, 2009). However, invasive prosthetic devices are the biggest risk factor for invasive *S. aureus* infections and bacteremia (Jensen et al., 1999). Via the bloodstream, the bacteria can disseminate through the body and cause infections in the liver, heart, kidneys, bones and other tissues (David and Daum, 2010, Lowy, 1998, Thomer et al., 2016). The course of infection is shaped by the interplay of bacterial and host factors (Scherr et al., 2014, Surmann et al., 2015). *S. aureus* is able to adopt different phenotypes to cause these diverse infections by switching between the virulence factors that are expressed. Several signaling systems control the expression of virulence factors in response to the respective environment of the infection (Balasubramanian et al., 2017, Jenkins et al., 2015). Generally, septic bacteremia with high toxin production and chronic biofilm-associated infection (e.g. osteomyelitis) are the polar opposites on the spectrum of potential infection outcomes of *S. aureus* (Brady et al., 2008, Powers and Bubeck Wardenburg, 2014).

During acute infections, *S. aureus* uses secreted exotoxins to damage host cells by forming pores in the membrane. At least 6 different pore-forming exotoxins (α-toxin, γ-toxin, PVL, LukAB, LukED and LukMF) can be produced by *S. aureus* during infection in the bloodstream (Dal Peraro and van der Goot, 2016, Otto, 2014, Vandenesch et al., 2012). The toxins lyse leukocytes, erythrocytes, the endothelial cells lining the blood vessels and epithelial tissues. Additionally, the non-pore forming β-toxin contributes to *S. aureus* pathogenesis (Huseby et al., 2007). These factors are upregulated during early bacteremia compared to asymptomatic nasal colonization indicating an adjustment to the changed environment (Jenkins et al., 2015). As the bloodstream is usually a sterile compartment, the presence of bacteria elicits an immune response that is counteracted with different immune evasion strategies (Fig. 1) (Goldmann and Medina, 2017, McGuinness et al., 2016, Thammavongsa et al., 2015). The chemotaxis inhibiting protein (CHIPS) is secreted to prevent the chemotaxis of neutrophils to the site of infection (de Haas et al., 2004, Postma et al., 2004). Reactive oxygen species-neutralizing compounds, e.g. staphyloxanthin and catalase, also ward off neutrophil attacks (Lan et al., 2010, Mandell, 1975). Nuclease is a secreted factor targeting and breaking traps of extracellular DNA (eDNA) produced by neutrophils to bind pathogens and create a milieu with high local concentrations of antimicrobials (Berends et al., 2010, Brinkmann et al., 2004). Breaking of these neutrophil extracellular traps additionally produces deoxyadenosine, a substance that triggers apoptosis in immune cells (Thammavongsa et al., 2009, Thammavongsa et al., 2013). Hence, the weapons of the neutrophils against the bacteria get turned against themselves. Similarly, the staphylococcal complement inhibitor (SCIN) and aureolysin, a secreted protease that cleaves complement factors, inhibit the opsonization by the complement system (Jongerius et al., 2007, Laarman et al., 2011).

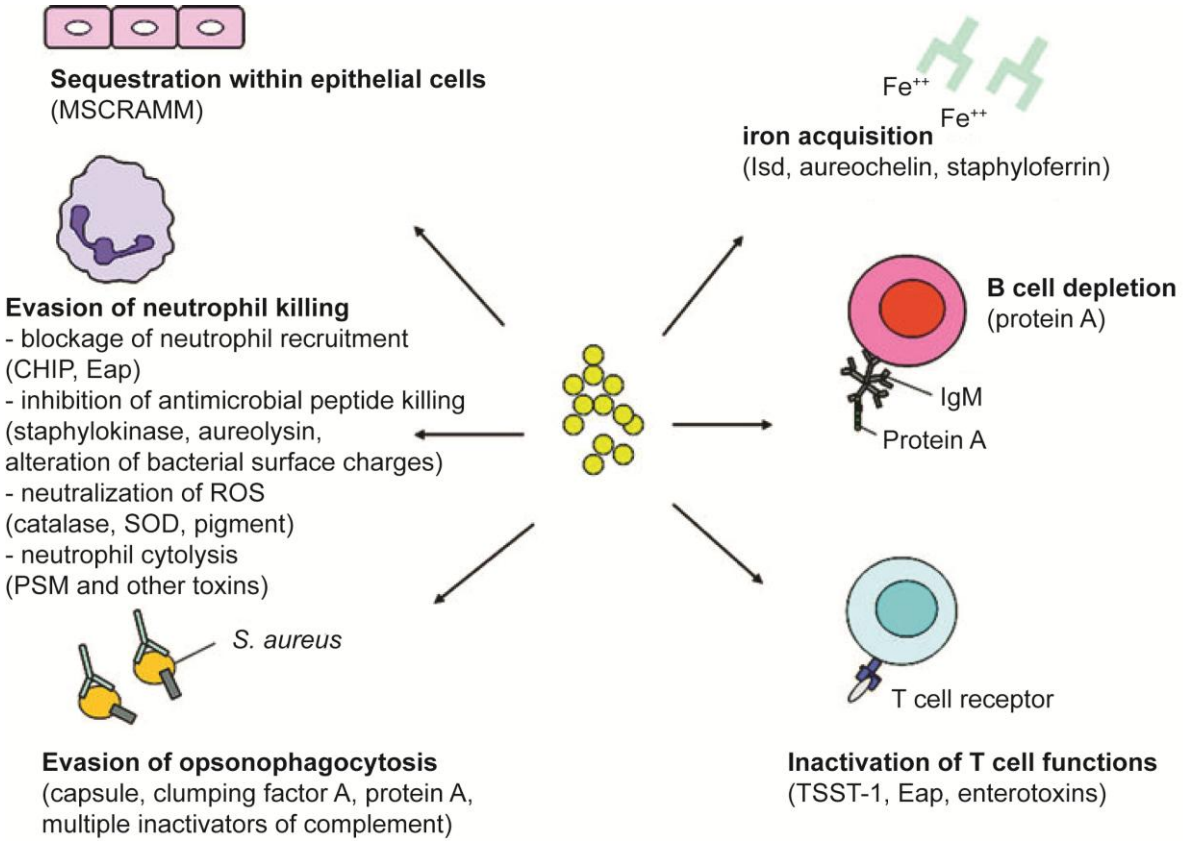

Fig. 1: *S. aureus* immune evasion strategies

S. aureus uses secreted and surface-associated virulence factors to escape the innate and adaptive immune system of the host. Figure adapted from (Liu, 2009) with permission of the publisher.

S. aureus-colonized individuals have high antibody titers against staphylococcal antigens but are not protected from infection as the pathogen also carries evasion strategies against the adaptive immune system (Dryla et al., 2005, Verkaik et al., 2009). The virulence factors toxic shock syndrome toxin-1 (TSST-1) and staphylococcal protein A (SpA) are superantigens against T-cells and B-cells, respectively (McCormick et al., 2001, Rahimpour et al., 1999, Silverman and Goodyear, 2006). They trigger a massive proliferation of immune cells, release of cytokines (TSST-1), and subsequent immune cell death (Goodyear and Silverman, 2004, Krakauer, 1999). At the same time, up to 30% of the circulating antibodies bind to SpA, thus reducing the capacity of an immune response against other staphylococcal antigens (Pauli et al., 2014, Silverman et al., 1993). Additionally, SpA can bind the Fc region of antibodies, thus preventing opsonophagocytosis and elimination by immune cells (Pauli et al., 2014).

To infect non-endothelial tissues, *S. aureus* must escape from the bloodstream by adhering to and invading the endothelium (Edwards and Massey, 2011, Pöhlmann-Dietze et al., 2000, Viegas et al., 2011). A large group of cell wall-associated proteins for adhesion to host structures is clustered as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Patti et al., 1994). MSCRAMMs specifically bind host receptors and are required for internalization of bacteria. Immune evading factors like SpA, clumping factors A and B (ClfA, ClfB) and the collagen-adhesin (Cna) are also part of the MSCRAMM family (Kang et al., 2013, McDevitt et al., 1997, Ni Eidhin et al., 1998, Patti et al., 1995). MSCRAMM-mediated adhesion is the initial step for biofilm formation (Heilmann, 2011). Generally, toxin production is highest during acute infection but toxins also play a role in the establishment of persistent infections. The type VII secretion system secretes small toxic peptides that are required for the formation of abscesses in chronic infections (Burts et al., 2008, Burts et al., 2005, Wang et al., 2016). Some toxins play dual roles in acute infections and the establishment of persistent infections in the form of biofilms. α-toxin mediates cell-to-cell contacts in biofilm formation (Caiazza and O'Toole, 2003). β-toxin can crosslink in the presence of the matrix component eDNA and thus improve biofilm formation (Doery et al., 1963, Huseby et al., 2007, Huseby et al., 2010). These dual functions for toxins in acute and chronic infections are exceptional examples and generally, gene expression varies considerably between bacteria in an acute compared to a chronic infection setting (Beenken et al., 2004, Garcia-Betancur et al., 2017). These changes are coordinated by major signaling networks including the quorum sensing system accessory gene regulator (Agr), the alternative sigma factor B (σ^B), the staphylococcal accessory regulator A (SarA) and the *S. aureus* exoprotein expression system (SaeRS) (Beenken et al., 2003, Beenken et al., 2010, Boles and Horswill, 2008, Mrak et al., 2012, Novick and Jiang, 2003, Rachid et al., 2000, Tuchscherr et al., 2015).

I.2 Biofilm formation in *S. aureus*

I.2.1 Structural components of *S. aureus* biofilms

Biofilm formation in *S. aureus* is closely interlinked with chronic or recurring infections (Costerton et al., 1999, Gonzalez et al., 2017, Harris and Richards, 2006, Parsek and Singh, 2003). Implanted foreign bodies, e.g. pacemakers or artificial joints, are particularly prone to become colonized by biofilm-forming bacteria but damaged host tissue, e.g. wounds or the lungs of cystic fibrosis patients, can also be affected by biofilms (Kirketerp-Moller et al., 2008, Marrie et al., 1982, Omar et al., 2017, Pawlowski et al., 2005, Wadstrom, 1989). This clinical relevance has sparked intense research in the regulatory pathways and mechanisms that control biofilm formation. This section will describe the stages of biofilm formation and the composition of sessile *S. aureus* communities.

Generally, biofilm formation consists of three stages: adhesion of cells to the surface; maturation of the biofilm; dispersion of the biofilm (Otto, 2013). Cell wall-associated proteins mediate the initial adhesion to host tissues or artificial surfaces coated with host matrix proteins (Patti et al., 1994). Teichoic acids (TA) are carbohydrate polymers that are anchored in the cell membrane (lipid teichoic acids, LTA) or cell wall (wall teichoic acids, WTA) and provide a protein-independent adhesion mechanism to artificial surfaces (Gross et al., 2001, Heptinstall et al., 1970, Xia et al., 2010). During maturation of the biofilm, the bulk of the extracellular matrix (ECM) is produced which encapsulates the cells of the biofilm. Hence, the biofilm biomass accumulates during this stage. The characteristic 3D-structure of biofilms is also shaped during the maturation phase (Otto, 2008, Otto, 2013, Periasamy et al., 2012). During dispersal, the ECM can become partially degraded by nucleases and proteases to facilitate dissemination of cells from the biofilm (Boles et al., 2010, Kiedrowski et al., 2011, Marti et al., 2010, Mrak et al., 2012). *S. aureus* does not produce enzymes capable of degrading its matrix polysaccharide but it is susceptible to glycoside hydrolases produced by other organisms (Fleming et al., 2017, Otto, 2013, Waryah et al., 2017).

S. aureus can use diverse structural elements for biofilm formation, namely exopolysaccharides, proteins and eDNA. The first component that was described to be involved in biofilm formation was the exopolysaccharide poly-N-acetylglucosamine (PNAG) also known as polysaccharide intercellular adhesin (PIA) (Cramton et al., 1999). The PNAG production machinery was originally described in *Staphylococcus epidermidis* but it is well conserved in *S. aureus* (Cramton et al., 1999, Heilmann et al., 1996). PNAG is produced by the genes of the *ica* operon (intercellular adhesion), *icaADBC.* Additionally, the *ica* locus contains a repressor, *icaR*, that is divergently transcribed from *icaADBC* and suppresses transcription of *ica* by binding to the promoter (Cerca et al., 2008, Jefferson et al., 2004). The proteins IcaA, IcaD and IcaC are membrane-associated, IcaB is an extracellular protein and IcaR is an intracellular protein. IcaA is a N-acetylglucosamine transferase that synthesizes the PNAG polymer from uridine diphosphate (UDP) N-acetylglucosamine monomers (Gerke et al., 1998). IcaD increases the efficiency of the reaction that leads to the polymerization of PNAG but its exact function remains elusive (Gerke et al., 1998). PNAG is translocated across the membrane by IcaC. Outside the cell, PNAG becomes partially deacetylated by IcaB giving it a positive net charge. This modification is important for the functionality of PNAG as it improves the attachment of the polymer to the cell surface (Vuong et al., 2004). The classification of *S. aureus* biofilm formation strategies into *ica*-dependent and *ica*-independent strategies highlights the principal role that PNAG holds in this process.

Ica-independent biofilm strategies were discovered by the lack of correlation between *ica*-expression and biofilm formation in several clinical isolates (Fitzpatrick et al., 2005, Toledo-Arana et al., 2005). Various proteinaceous components have been found to contribute to biofilm formation. *S. aureus* encodes a plethora of surface-associated proteins involved in intercellular or surface adhesion. Among them are members of the aforementioned MSCRAMM protein family, e.g. Bap, SasG, SasC and SpA (Cucarella et al., 2001, Heilmann, 2011, Merino et al., 2009, Schroeder et al., 2009). Unlike the covalently surface-linked MSCRAMMs, secretable expanded repertoire adhesive molecules (SERAM) are secreted from the cell and bind the cell surface non-covalently (Chavakis et al., 2005). The

extracellular adherence protein (Eap) and extracellular matrix protein-binding protein (Emp) are members of this protein family and contribute to biofilm formation under iron-depleted conditions (Johnson et al., 2008). Proteins in the ECM have additional functions beyond adhesion as exemplified by a group of 7 peptides termed phenol-soluble modulins (PSM) (Mehlin et al., 1999). The multifunctional PSM family consists of PSMα1-4, PSMβ1-2 and the RNAIII-encoded δ-toxin (Cheung et al., 2014, Verdon et al., 2009, Wang et al., 2007). PSM do not require a signal sequence for secretion because they are exported by a PSM-specific export system. The export system also confers immunity against the antimicrobial effects of PSM (Chatterjee et al., 2013). PSM interact with the immune system in several ways. The peptides have strong cytolytic activity against leukocytes (Wang et al., 2007). Additionally, PSM aid intracellular survival of *S. aureus* in professional and non-professional phagocytes and modulate the response of the immune system (Grosz et al., 2014, Kretschmer et al., 2011, Schreiner et al., 2013). PSM have surfactant-like properties that facilitate the spreading of the non-motive staphylococcal cells over wet surfaces (Kizaki et al., 2016, Periasamy et al., 2012, Tsompanidou et al., 2013). The effect of PSM on biofilm formation depends on their oligomeric state (Marinelli et al., 2016). Monomeric PSM with the above-mentioned surfactant-like properties contribute to dissemination of cells from the biofilm, thus affecting the biofilm biomass negatively (Periasamy et al., 2012, Wang et al., 2011). However, as dissemination of cells from the biofilm occurs only locally, it is a beneficial strategy for the bacteria because it enables secondary infections in other sites (Le et al., 2014, Otto, 2013, Yarwood et al., 2004).

Remarkably, PSM can also polymerize and form long fibers with an unusual α-helix structure (Schwartz et al., 2012, Tayeb-Fligelman et al., 2017). These fibers potentially contribute to the integrity of biofilms (Schwartz et al., 2012). Due to the fibrillar structure of the PSM fibers, it was suggested that PSM can fold into functional amyloids (Schwartz et al., 2015, Schwartz et al., 2012). Amyloid fibers are an abundant component of the ECM in *Bacillus subtilis* and *Escherichia coli* biofilms in the form of TasA and curli fibers, respectively (Barnhart and Chapman, 2006, Romero et al., 2010). Unlike the amyloid fibers formed by the protein Bap,

which is only found in bovine mastitis isolates, the presence of amyloid folds in PSM fibrils is disputed (Cucarella et al., 2001, Di Martino, 2016, Taglialegna et al., 2016). It has been argued that the PSM fibrils that were interpreted as amyloid fibers do not actually depend on an amyloid fold of the peptides but result from non-specific interactions between PSM and eDNA (Joo et al., 2016, Zheng et al., 2017).

The biofilm ECM also contains proteins that – unlike the proteins of the MSCRAMM family – are not functionally dedicated to adhesion or cell-to-cell interaction. Abundant cytoplasmic proteins get recycled to build up the ECM under acidic conditions (Foulston et al., 2014).

Thirdly, eDNA is a major structural component of the ECM. Initially, it was deemed to be a waste product from lysed cells but now it is recognized that cell lysis and eDNA release occur in a controlled manner (Flemming and Wingender, 2001, Flemming and Wingender, 2010, Mann et al., 2009). The holin-like and antiholin-like proteins CidA and LrgA affect cell lysis, eDNA release and biofilm formation negatively and positively, respectively (Patton et al., 2005, Rice et al., 2007). Holins act by forming pores in the membrane and activating hydrolases that attack the cell wall (Rice et al., 2003). The antiholin LrgA acts by inhibiting the function of the holin CidA (Ranjit et al., 2011, Rice et al., 2003). The plurality of lysis-controlling system indicates a strict regulation in response to intracellular and extracellular cues (Sadykov and Bayles, 2012).

The complexity and dynamics of the ECM are further increased by interactions between different matrix components (Payne and Boles, 2015). It was demonstrated that eDNA can interact with cytoplasmic proteins and PSM but the physiological relevance of the latter interaction remains to be determined (Dengler et al., 2015, Foulston et al., 2014, Joo et al., 2016, Zheng et al., 2017). Furthermore, PNAG-WTA-interplay due to electrostatic interactions was found despite earlier reports that WTA are not required to link PNAG to the cell surface (Formosa-Dague et al., 2016, Vergara-Irigaray et al., 2008).

The biofilm-associated lifestyle of *S. aureus* has consequences beyond the synthesis of ECM products as it leads to adjustments of the general cell physiology. Comparative studies of planktonic and biofilm-bound cells showed differences between the lifestyles regarding gene expression, the proteome and the lipid composition of the membrane (Beenken et al., 2004, Dubois-Brissonnet et al., 2016, Islam et al., 2014, Resch et al., 2006, Resch et al., 2005b). The growth rate of bacteria in a biofilm is reduced in comparison to planktonic bacteria (Hodgson et al., 1995). The metabolism is switched from aerobic to mostly anaerobic energy generation in biofilms (Beenken et al., 2004, Resch et al., 2005b). Importantly, these metabolic changes do not occur uniformly throughout the community but biofilm populations are highly heterogeneous (Rani et al., 2007, Stewart and Franklin, 2008, Wimpenny et al., 2000). The metabolic profiles of the cells appear stratified in response to the gradients of nutrients and oxygen that are found in the biofilm (Rani et al., 2007). The gradients result from the limited diffusion of substances like oxygen that are also continuously used by the bacteria (Characklis et al., 1990, Werner et al., 2004). An extreme example of metabolic heterogeneity are dormant persister cells that make up up to 1% of the biofilm population (Lewis, 2008).

I.2.2 Regulation of *S. aureus* biofilm formation

Analogous to the diverse structural elements related to biofilm formation, *S. aureus* also has a collection of tightly interlinked regulatory systems for the process (Beenken et al., 2010, Bischoff et al., 2001, Heinrichs et al., 1996). This section will highlight two prominent regulators of biofilm formation: Agr and σ^B.

The **accessory gene regulator (Agr)** is a quorum sensing (QS) system that controls gene expression in response to the population density (Fig. 2) (Recsei et al., 1986). The *agr* locus in the chromosome contains two divergent promoters that control the two central components of the system: the P2 promoter in front of the QS system operon *agrBDCA* and the P3 promoter in front of the effector small RNA (sRNA) RNAIII (Koenig et al., 2004, Peng et al., 1988). The QS system consists of a two-component system (TCS) with a histidine

kinase (HK) with an extracellular sensor domain (AgrC) and its cognitive response regulator (RR) (AgrA), a small peptide (AgrD) that is the precursor of the signaling molecule and a peptidase (AgrB) that processes AgrD into the mature signaling molecule and secretes it from the cell (Thoendel et al., 2011). The mature version of the signaling molecule, called autoinducing peptide (AIP), consists of 7-10 amino acids encoded in the middle of *agrD* (Ji et al., 1995). AIP is a stable peptide with a lifetime of 3 h due to a thiolactone ring that is formed between the C-terminal amino acid and a conserved cysteine (Chan et al., 2004, Wright et al., 2005). Four Agr-types with variations in the amino acid sequence of AIP have been found in *S. aureus* (Ji et al., 1997). The name AIP refers to the function of the molecule as its own inducer. Upon sensing of AIP, AgrC undergoes autophosphorylation and subsequently transfers a phosphate group to AgrA (Lina et al., 1998). This activation of the kinase activity of AgrC only occurs after binding of the cognate AIP-type, binding of different AIP-types results in AgrC inhibition instead (Geisinger et al., 2009, Ji et al., 1997). AgrA~P acts as a response regulator and induces expression from P2 and P3. The P3-transcript RNAIII is a 514 nt regulatory RNA that additionally contains the coding transcript for δ-toxin (Novick et al., 1993). RNAIII is a post-transcriptional regulator that can have positive or negative effects on translation depending on the target (Boisset et al., 2007, Huntzinger et al., 2005, Novick et al., 1993). Interestingly, the promoters P2 and P3 are not activated simultaneously (Garcia-Betancur et al., 2017, Xiong et al., 2002). Activation of P2 occurs earlier because AgrA binds it with higher affinity (Koenig et al., 2004). On the other hand, once P3 becomes activated, its activity is higher than that of P2.

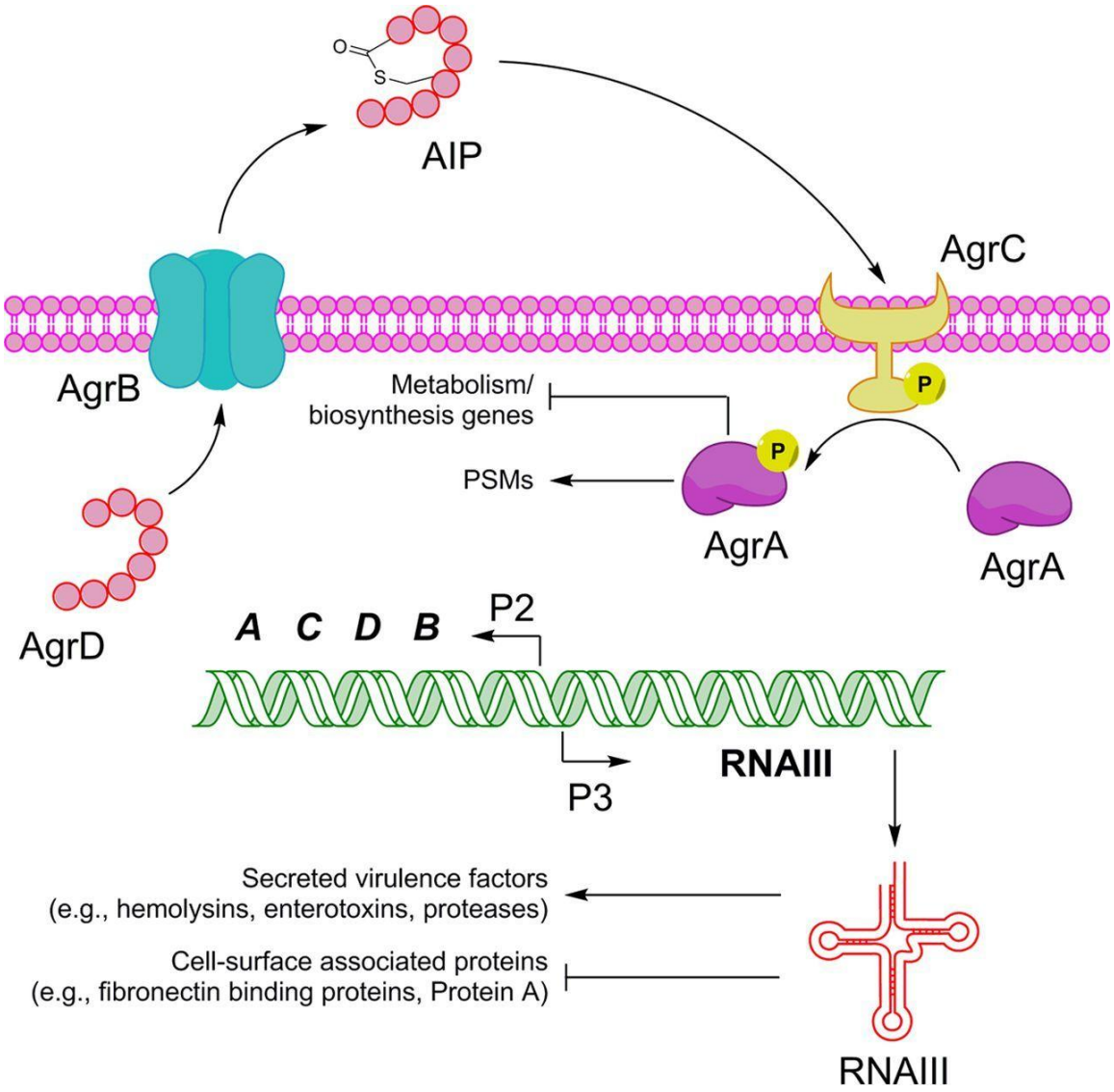

Fig. 2: The accessory gene regulator of *S. aureus*

Schematic representation of the genetic organization of *agr*, AIP-dependent autoinduction and RNAIII-dependent and –independent gene regulation. Image credits: (Salam and Quave, 2018), published under CC BY 4.0.

Agr is a global regulator in *S. aureus* with many genes related to virulence and biofilm formation in its regulon. The response regulator AgrA directly controls the expression of *agrBDCA* and a small number of additional genes including the PSM (Queck et al., 2008). The majority of genes in the Agr regulon is regulated by RNAIII. SpA and α-toxin are examples for negatively and positively regulated targets of RNAIII, respectively (Fig. 2). The negative effect on translation of *spa*-mRNA occurs due to a duplex between RNAIII and the ribosome binding site (RBS) on the mRNA which prevents translation initiation (Huntzinger et al., 2005). In contrast, RNAIII causes a conformational change of *hla*-mRNA which makes

the RBS accessible and results in translation of the α-toxin (Morfeldt et al., 1995, Novick et al., 1993). Besides direct effects on target translation, RNAIII also exerts indirect effects. The repressor of toxins (Rot) is a major effector downstream of RNAIII. As the name implies, Rot represses the transcription of secreted toxins and other virulence factors (McNamara et al., 2000, Said-Salim et al., 2003). By repressing translation of Rot, RNAIII has a positive, albeit indirect, effect on the production of these virulence factors (Boisset et al., 2007, Geisinger et al., 2006). Rot is considered a positive regulator of biofilm formation because it represses proteases that degrade proteinaceous components of the ECM and upregulates adhesins including SpA (Mootz et al., 2015, Said-Salim et al., 2003).

Overall, activation of Agr has a negative effect on early stages of biofilm formation because it suppresses surface adhesins required for the attachment phase (Recsei et al., 1986). The expression of *ica* is independent of Agr regulation (Vuong et al., 2000). In late stages of biofilm formation, the effect of Agr regulation is ambiguous. The QS system boosts the production of proteases and monomeric PSM that lead to dissemination of cells from the biofilm but on the other hand oligomeric PSM stabilize the ECM (Boles and Horswill, 2008, Schwartz et al., 2012).

The autoinducing properties of Agr imply that the system is predominantly regulated by the density of cells in a given space and its activity is coupled to the growth phase *in vitro*. The dimensions of biofilms often surpass the calling distance of QS systems which ranges between 10 and 100 µm (Strassmann et al., 2011). Therefore, Agr is not homogenously active in *S. aureus* biofilms but is also subject to heterogeneity (Garcia-Betancur et al., 2017, Yarwood et al., 2004). Additionally, the system is fine-tuned by other regulators including SarA, σ^B (see below) and SrrAB (see chapter I.3.2) (Heinrichs et al., 1996, Horsburgh et al., 2002, Lauderdale et al., 2009, Pragman et al., 2004, Rechtin et al., 1999, Yarwood et al., 2001).

The **stress-induced sigma factor σ^B** is the best characterized of the three alternative sigma factors that control transcription initiation in *S. aureus* alternatively to the primary sigma

factor σ^A. The alternative sigma factors σ^B, σ^H and σ^S play roles in stress response, competence and pathogenicity, respectively (Deora and Misra, 1996, Morikawa et al., 2003, Shaw et al., 2008, Wu et al., 1996). Of the alternative sigma factors, the stress-induced sigma factor σ^B is best characterized. The sigma factor σ^B is held in an inactive state by the anti-sigma factor RsbW (Miyazaki et al., 1999). Upon the sensing of stress conditions, the anti anti-sigma factor RsbV becomes de-phosphorylated by the phosphatase RsbU and competes with σ^B for RsbW binding (Giachino et al., 2001, Palma and Cheung, 2001). Through this process the anti-sigma factor releases σ^B, which is free to bind the RNA polymerase and initiate transcription of σ^B-dependent genes. The direct and indirect regulon of σ^B contains more than 200 up- or down-regulated genes (Bischoff et al., 2004, Gertz et al., 2000). The control of σ^B activation is well-studied in *B. subtilis* and RsbV, RsbW and σ^B are conserved between *B. subtilis* and *S. aureus*. However, in comparison to *B. subtilis*, the *S. aureus* operon is truncated and missing several genes for stress-sensing proteins involved in activation of the phosphatase RsbU (Pane-Farre et al., 2009, Wu et al., 1996). Therefore, the molecular mechanism behind σ^B activation and how RsbU perceives stress remains enigmatic.

Despite the uncertainty regarding the activation of σ^B, several factors influencing its activity have been identified. The activity of σ^B is highest in the late exponential growth phase and quickly decreases in the stationary phase (Giachino et al., 2001, Kullik and Giachino, 1997). Other stimuli for σ^B include acidic or alkaline pH, high salt concentrations, ethanol and heat shock (Chan et al., 1998, Giachino et al., 2001, Kullik and Giachino, 1997). Importantly, disturbances in cell wall structure or synthesis also activate σ^B (Morikawa et al., 2001).

σ^B is an important factor for the establishment of persistent infections and a positive regulator of biofilm formation (Atwood et al., 2015, Lauderdale et al., 2009, Rachid et al., 2000, Tuchscherr et al., 2015). Early stages of biofilm formation are positively influenced by σ^B through its effect on the expression of the adhesive factors ClfA and fibronectin binding protein A (FnbA) (Entenza et al., 2005). σ^B also represses proteases that degrade

proteinaceous components of the ECM (Marti et al., 2010). The effect of σ^B on *ica*-dependent biofilm formation is more complex. Initially, it was reported that σ^B positively affects expression of the *ica* operon (Rachid et al., 2000). Later findings point towards a subordinate role of σ^B for *ica* expression and SarA as the major regulator (Valle et al., 2003). Additionally, σ^B can modulate *ica* expression in an indirect manner through the repressor IcaR (Cerca et al., 2008). The recent discovery of an additional regulator of *ica* shows that the genetic regulation of PNAG production is an ongoing field of research (Yu et al., 2017).

More than 200 genes are regulated in a σ^B-dependent manner, but only 23 genes have the σ^B binding motif ($GTTTTAN_{14}TGGAAA$) in their promoter (Bischoff et al., 2004, Gertz et al., 2000). The majority of the σ^B regulon is controlled indirectly via the modulation of other regulatory systems. Notably, σ^B is an antagonist of Agr but it is unclear how the effect on the quorum sensing system is exercised (Bischoff et al., 2001, Mootz et al., 2015, Shaw et al., 2006). By contrast, the promoter of *sarA* – considered a master regulator of biofilm formation – contains a σ^B binding site for direct upregulation of the operon (Bayer et al., 1996, Beenken et al., 2003, Bischoff et al., 2001, Rechtin et al., 1999).

I.2.3 Model systems in biofilm research

Biofilm research relies on *in vitro* model systems that provide reproducible experimental conditions. The studies that identified the regulators and components of *S. aureus* biofilms were performed on **submerged biofilms** – surface-attached aggregates of bacteria covered in growth medium. Submerged biofilm models can use static growth conditions or dynamic conditions with a continuous flow of growth medium (Beenken et al., 2004, Islam et al., 2014, Merritt et al., 2005). Several external factors induce biofilm formation under laboratory conditions and are therefore added to growth media to study biofilm formation. Most commonly, NaCl and glucose are added to biofilm medium, but ethanol, magnesium, human plasma and subinhibitory concentrations of antibiotics also promote biofilm formation (Chen et al., 2012, Kaplan et al., 2012, Koch et al., 2014b, Lim et al., 2004, Rode et al., 2007, Waldrop et al., 2014). The mode of action for some of the medium additives is known. For

instance, metabolic products of glucose consumption lower the pH of the medium and thereby inhibit Agr (Regassa et al., 1992). Glucose-responsive proteins that affect *ica* transcription could contribute to the positive effect on biofilm formation (You et al., 2014). Biofilm formation is response to high salt concentrations is dependent on the action of σ^B (Rachid et al., 2000). Plasma proteins enhance biofilm formation by coating artificial surfaces and thus improving the adhesion of *S. aureus* to the surface (Francois et al., 1996).

Alternative laboratory models for biofilm formation have been used for other bacterial species. *B. subtilis* forms robust floating **pellicle biofilms** at the air-liquid interface of static cultures (Kobayashi, 2007, Vlamakis et al., 2013). The **macrocolony biofilm** model uses biofilms grown on a nutrient agar surface at the agar-air interface (Serra and Hengge, 2014). Use of the macrocolony model has led to significant progress in the understanding of processes related to three dimensional (3D) organization of biofilms and heterogeneity in biofilms in *B. subtilis*, *E. coli* and *Pseudomonas aeruginosa* (Branda et al., 2001, Dietrich et al., 2013, Romero et al., 2010, Serra et al., 2013a, Serra et al., 2013b, Vlamakis et al., 2008, Yang et al., 2011). Macrocolony biofilms are characterized by wrinkles and folds on the colony surface that represent a macroscopic semi-quantitative readout for the production of ECM (Branda et al., 2006, Ray et al., 2012). The wrinkled surface increases the surface-to-volume ratio of the macrocolony, hence increasing the zones where aerobic metabolism is possible (Dietrich et al., 2008, Kolodkin-Gal et al., 2013). The wrinkles occur when lateral pressure produced by the growing community is released at the sites of locally confined cell death and they are considered a community-wide stress response (Asally et al., 2012). Spatial heterogeneity of cells in a biofilm based on physico-chemical gradients in the environment has been studied in several examples. In *B. subtilis*, spore formation – a form of bacterial differentiation – primarily occurs in the protruding wrinkles of the macrocolony (Branda et al., 2001). *B. subtilis* develops distinct cell lineages with a defined fate based on the spatial localization within the macrocolony (Vlamakis et al., 2008). In *E. coli* macrocolonies, ECM production throughout the colony is stratified (Serra et al., 2013b). Cells in the oxygen-depleted but nutrient-rich lower layers produce an ECM that contains flagella,

whereas the ECM of the upper layers is characterized by amyloid curli fibers and cellulose (Serra et al., 2015). Disturbances of the ECM stratification negatively impact the integrity of the colony (Serra et al., 2015). Studies of macrocolony aggregates in *B. subtilis*, *E. coli* and *P. aeruginosa* were performed on MSgg agar, salt-free LB agar and tryptone agar, respectively (Branda et al., 2001, Dietrich et al., 2013, Serra et al., 2013a).

Recently, a macrocolony biofilm model for *S. aureus* has been developed. Growth conditions on a complex medium agar that is supplemented with magnesium trigger the aggregation into a 3D-structured macrocolony (Koch et al., 2014b). Host tissues that are rich in magnesium, e.g. kidneys and bones, are important niches for persistent infections with *S. aureus* (Brady et al., 2006, Cheng et al., 2009, Jahnen-Dechent and Ketteler, 2012, Koch et al., 2014b). Magnesium plays a central role in the genetic cascade that controls the formation of macrocolony aggregates in *S. aureus* (Garcia-Betancur et al., 2017). Magnesium ions from the medium bind to the negatively charged teichoic acids (TA) in the cell wall (Heckels et al., 1975, Heckels et al., 1977, Heptinstall et al., 1970, Lambert et al., 1975a, Lambert et al., 1975b). This reduces electrostatic repulsions between the TA and creates a stable network which increases the rigidity of the cell wall. The magnesium-induced change of the cell wall properties is an environmental cue to activate σ^B-dependent changes of gene expression including the repression of Agr (Gertz et al., 2000, Kullik et al., 1998, Kullik and Giachino, 1997, Rachid et al., 2000). This relay of gene expression changes is an essential signaling cascade for macrocolony biofilm formation in *S. aureus* (Fig. 3) (Garcia-Betancur et al., 2017).

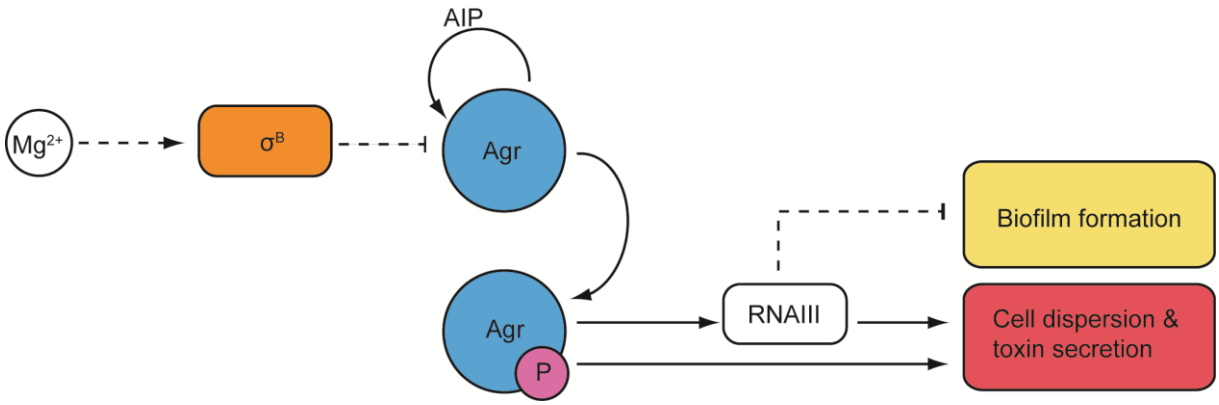

Fig. 3: Signaling cascade in the macrocolony biofilm model

Magnesium in the growth medium activates σ^B, which represses Agr. Autoactivated Agr and RNAIII activate cell dispersion and repress biofilm formation. Continuous and dashed lines indicate direct and indirect effects, respectively. Scheme adapted from (Garcia-Betancur et al., 2017) published under CC BY 4.0.

Similarly to the occurrence of distinct lineages in *B. subtilis* biofilms, differentiation into subpopulations with defined cell fates is also found in the macrocolony model of *S. aureus* (Garcia-Betancur et al., 2017). Two distinct cell lineages with gene expression patterns related to biofilm formation and biofilm dissemination develop in the macrocolony biofilms. The emergence of the cell lineages is mediated by a bistable switch of Agr in response to external conditions as the biofilm formation-related lineage only occurs in the presence of magnesium (Garcia-Betancur et al., 2017). Within the macrocolony aggregate, fluorescent genetic markers that are associated with the respective lineages are expressed in different regions (Garcia-Betancur et al., 2017). In addition to diversification of a transcriptional level, the macrocolony model of *S. aureus* has been used to demonstrate that intrastrain competition can lead to diversification on a genetic level (Koch et al., 2014b). Adaptation and counter-adaptation lead to the emergence of two new strains with fitness benefits over the parent strain. The point mutations that occur in the first adapted strain affect the *sigB* operon and alter the production of bacteriocins and PSM. The second emerging counter-adapted strain is resistant to the action of the bacteriocins due to an altered cell wall composition (Koch et al., 2014b). Similar transcriptional and genetic adaptations of *agr* and *sigB* are also found in clinical isolates, showing that the macrocolony model can be a suitable approach to

study to processes during *S. aureus* pathogenesis and biofilm formation *in vivo* (Inose et al., 2006, Traber et al., 2008).

I.3 Novel regulatory pathways in the macrocolony model

This section of the doctoral thesis introduces three well-characterized metabolic and regulatory pathways of *S. aureus* that have been assigned a novel function as regulators of macrocolony morphology in this thesis.

I.3.1 Purine biosynthesis

The purine bases adenine and guanine fulfill a plethora of functions in bacterial cell biology. Most prominently they are coupled to ribose or 5'deoxyribose and incorporated into RNA (adenosine and guanosine) or DNA (deoxyadenosine and deoxyguanosine), respectively. Additional functions for the purines include energy carriers (ATP, GTP), signaling molecules (c-di-AMP, (p)ppGpp), enzyme cofactors (riboflavin, folate) and phosphoryl group donors (Brown and Williamson, 1982, Corrigan et al., 2011, Corrigan et al., 2015, Crosse et al., 2000, Karaolis et al., 2005, Merkler and Schramm, 1987). Purine biosynthesis is required for full virulence of *S. aureus* and contributes to resistance against the antibiotic rifampicin (Connolly et al., 2017, Lan et al., 2010, Yee et al., 2011).

The *de novo* synthesis of purines occurs attached to the molecule ribose-5-phosphate (Hartman and Buchanan, 1959). The purine bases adenine and guanine initially share a common biosynthetic pathway that is branched at the molecule inosine monophosphate (IMP). In *S. aureus*, 11 enzymatic reactions and 5 ATP molecules are necessary to synthesize IMP from 5-phosphoribosyl-pyrophosphate (PRPP) (Zhang et al., 2008). The genetic organization of the involved genes is well conserved from *Enterobactericeae*, but the greatest similarity is found to *B. subtilis* (Baxter-Gabbard and Pattee, 1970, Ebbole and Zalkin, 1987, Ebbole and Zalkin, 1989, Fox et al., 2007). The majority of the genes is found in the 11 kDa operon *purEKCSQLFMNHD*. Additionally, the genome of *S. aureus* contains the associated genes *purA*, *purB* and the repressor *purR* (Baxter-Gabbard and Pattee, 1970). Interestingly, two versions of the gene *purL* are found in prokaryotes. Gram-negative

bacteria have a large copy of *purL* similar to eukaryotes. In contrast, gram-positive bacteria have a short copy of *purL* and the additional genes *purQ* and *purS* (Hoskins et al., 2004).

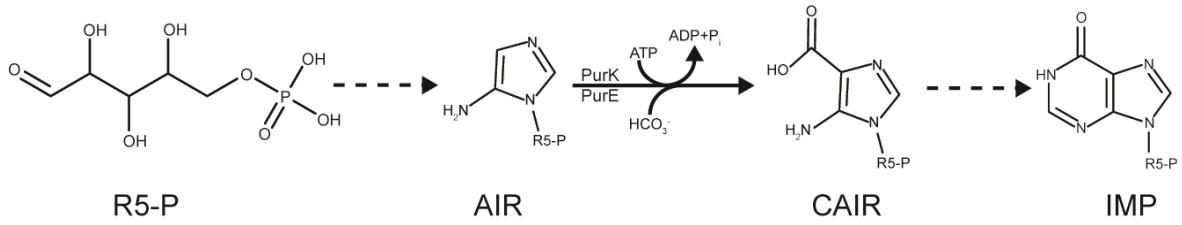

Fig. 4: PurEK mediated reactions in purine biosynthesis

R5-P is processed to AIR in several reaction steps. PurEK mediate the two-step reaction of AIR to CAIR, which is further processed to IMP.

Another particularity in prokaryotes compared to higher eukaryotes is the gene *purK*. In vertebrates, the reaction from 5-aminoimidizole ribonucleotide (AIR) to 5'-phosphoribosyl-4-carboxy-5-aminoimidazole (CAIR) is performed by the enzyme PurE. In prokaryotes, two separate reactions are responsible for the process. In the first step, PurK catalyzes the reaction from AIR to N5-carboxy-aminoimidazole ribonucleotide (NCAIR), an instable reaction intermediate. In this reaction, bicarbonate is ligated to AIR in an ATP-dependent manner. A mutase reaction mediated by PurE is responsible for the conversion from NCAIR to CAIR (Fig. 4) (Mueller et al., 1994). Closing of the purine ring occurs downstream of this step in the reaction that results in IMP, the last common precursor for adenine and guanine (Hartman and Buchanan, 1959).

Purine biosynthesis is regulated by the availability of precursors and products of the pathway. High levels of PRPP positively affect purine biosynthesis whereas the presence of IMP, adenine or guanine inhibits the process (Baxter-Gabbard and Pattee, 1970). This regulatory mechanism is also found in *B. subtilis* where the mode of action has been solved. PRPP sequesters the repressor PurR so that purine biosynthesis-related genes can be transcribed (Weng et al., 1995). Independently of the availability of the purine metabolites, the *pur* operon in *S. aureus* is repressed by high temperatures and in a mutant of the serine-threonine kinase PknB (Donat et al., 2009, Fleury et al., 2009).

I.3.2 Oxygen sensing

The size of biofilms varies from microcolonies with just a few micrometers diameter and thickness to macrocolonies that can have a diameter of several millimeters and a thickness of more than 100 µm (Drescher et al., 2016, Serra et al., 2013a). As it is not actively transported through the biofilm, oxygen must diffuse through the biofilm to be available to the cells (Stewart, 2003). The oxygen availability for cells far from the surface is further reduced by the active depletion of oxygen by metabolically active cells. The question how far oxygen can penetrate into biofilms by diffusion has been approached theoretically and practically. The maximum penetration depth of oxygen into *P. aeruginosa* biofilms was calculated to be 77 µm (Stewart, 2003). Measurements with a microelectrode showed that oxygen could not penetrate *P. aeruginosa* biofilms deeper than 60 µm (Dietrich et al., 2013). Similar measurements using microelectrodes could not detect oxygen in *S. aureus* biofilms at a depth of 50 µm (Kiamco et al., 2017). Alternatively, oxygen concentrations can be measured non-invasively using dyes whose fluorescence is quenched by oxygen. Using this method, anoxic microniches were detected in biofilms of *Pseudomonas putida* at a depth of 10 – 40 µm (Kuhl et al., 2007). Similarly, anoxic microniches that allow for the coexistence of obligate anaerobe bacteria in a biofilm of aerobe bacteria that deplete environmental oxygen were calculated to occur with a microcolony diameter of 25 µm (Stewart, 2003). These calculations and measurements show that bacteria frequently encounter anaerobic conditions in biofilms. Consequently, it was shown that active protein synthesis only occurs in the top 30 µm of *P. aeruginosa* biofilms (Xu et al., 1998).

As oxygen plays a central role in the metabolism as an electron acceptor in the respiratory chain, the switch from an aerobic to an anaerobic lifestyle leads to strong changes in gene expression. More than 200 genes, about 10% of the genome, were differentially expressed in *S. aureus* under anaerobic conditions compared to aerobic conditions (Fuchs et al., 2007). Similarly, the aerobic-anaerobic switch changed the expression of several hundred genes in *B. subtilis* (Ye et al., 2000).

S. aureus is a facultative anaerobe bacterium, capable of switching from aerobic to anaerobic metabolism. When oxygen abundance is sufficient, glycolysis is the preferred pathway to generate energy and pyruvate from glycolysis is fueled into the TCA cycle (Seidl et al., 2009). Oxygen is necessary as an electron acceptor to regenerate NADH to NAD+ (Ledala et al., 2014). In absence of oxygen, the metabolism is switched to fermentation producing lactic acid (Hall and Ji, 2013). Additionally, *S. aureus* can use nitrate and nitrite as alternative electron acceptors to regenerate the reducing agents derived from the TCA cycle (Burke and Lascelles, 1975, Fuchs et al., 2007, Schlag et al., 2008).

Different systems for oxygen sensing that have an impact on biofilm formation have evolved in bacteria to govern the metabolic switch between aerobic and anaerobic metabolism. The opportunistic gram-negative pathogen *Burkholderia dolosa* has an oxygen-responsive two-component system (Schaefers et al., 2017). The TCS FixLJ becomes activated by low oxygen levels, presumably the oxygen concentration is sensed directly by a PAS-domain in the membrane-bound histidine kinase FixL (Schaefers et al., 2017). The deletion mutant *ΔfixLJ* forms a stronger but less structured biofilm, but the mechanism behind the changes remains to be elucidated (Schaefers et al., 2017). *Shewanella putrefaciens* is capable of sensing oxygen by the diguanylate cyclase DosD (Wu et al., 2013). DosD produces the secondary messenger c-di-GMP in response to oxygen, thus affecting the production and export of an adhesion important for biofilm formation (Theunissen et al., 2010, Wu et al., 2013).

S. aureus contains 16 two-component systems for signal transduction into the cell. Three of these systems – AirSR, NreABC and SrrAB – are redox-sensitive (Schlag et al., 2008, Sun et al., 2012, Throup et al., 2001, Yarwood et al., 2001). NreABC controls the uptake of nitrate and nitrite under anaerobic conditions. The regulon contains 37 genes that are all related to the use of nitrate and nitrite as electron acceptors (Schlag et al., 2008). The histidine kinase AirS is inactive in the reduced form and gets activated by oxidation (Sun et al., 2012). The regulon of AirSR contains up to 355 genes in stationary phase and is functionally not limited

to metabolism but also affects virulence, antibiotic resistance, cell wall synthesis, DNA replication and protein synthesis (Sun et al., 2012, Yan et al., 2011). SrrAB was identified because of its high similarity with the redox-responsive TCS ResDE in *B. subtilis* (Nakano et al., 1996, Throup et al., 2001, Yarwood et al., 2001). SrrA was determined to be the intracellular response regulator that affects gene expression by binding to the DNA. It is dependent on the activation by the transmembrane histidine kinase SrrB (Throup et al., 2001, Yarwood et al., 2001). The genes are co-transcribed in an operon, but the response regulator *srrA* can also be transcribed independently (Yarwood et al., 2001). In contrast to AirS, SrrB becomes activated under anaerobe conditions (Pragman et al., 2004, Throup et al., 2001, Yarwood et al., 2001). The exact stimulus that activates SrrB was unknown for a long time until studies with mutants that have a defect in the respiratory chain shed some light. It has been established that SrrB responds to a pool of reduced menaquinone as it occurs when the respiratory chain is blocked in absence of an electron acceptor (Kinkel et al., 2013, Kohler et al., 2008, Mashruwala et al., 2017b, Schlievert et al., 2013). Activated SrrB can phosphorylate SrrA and SrrA~P binds to DNA to act as a transcriptional regulator (Throup et al., 2001, Yarwood et al., 2001). The SrrAB-regulon is subject to change depending on the strain background, growth phase and oxygen concentration (Pragman et al., 2007a, Pragman et al., 2007b, Pragman et al., 2004, Ulrich et al., 2007, Wilde et al., 2015). There is comprehensive data that SrrAB represses the production of toxins via *agrBDCA* and is capable of inducing *srrAB* expression in a positive feedback loop (Fig. 5) (Mashruwala and Boyd, 2017, Pragman et al., 2004, Wilde et al., 2015). Expression of *srrAB* is further repressed by the redox sensitive transcription factor Rex under aerobe conditions (Pagels et al., 2010). SrrAB induces genes that are required to detoxify reactive nitrogen species (Kinkel et al., 2013). Mutants of *srrAB* were found the be defective in biofilm formation but it is unclear whether the production of PNAG plays a role in this context (Mashruwala et al., 2017a, Mashruwala et al., 2017b, Tu Quoc et al., 2007, Ulrich et al., 2007). More recently, it was suggested that the biofilm defect of *ΔsrrAB* stems from the role

of SrrAB in the induction of programmed cell lysis, which leads to the release of eDNA under anaerobic conditions (Mashruwala et al., 2017b).

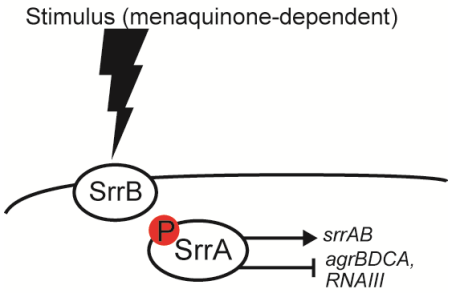

Fig. 5: SrrAB activation and activity in micro- and anaerobic conditions

Upon sensing of a menaquinone-dependent stimulus, SrrB autophosphorylates and transfers a phosphoryl group to SrrA, thus activating it. SrrA upregulates transcription of *srrAB* and represses *agrBDCA* and RNAIII.

I.3.3 Gluconeogenesis

Glucose is a prime energy and carbon source for many bacteria, including *S. aureus* (Monod, 1941). The central role of glucose is highlighted by the presence of four highly redundant glucose transporters (*glcA*, *glcB*, *glcC*, *glcU*) in the genome of *S. aureus*, two of which are exclusively found in this organism (*glcA*, *glcC*) (Vitko et al., 2016). However, the human skin, which is a natural niche of *S. aureus*, is low in free carbohydrates (Vitko et al., 2016). Therefore, *S. aureus*, as most other organisms, is capable of synthesizing glucose from other substrates by gluconeogenesis, to ensure the constant availability of glucose. The substrates include glucogenic amino acids, triglycerides and pyruvate as well as lactate and are derived from the main energy sources proteins, lipids and carbohydrates, respectively (Anderson and Wood, 1969, Halsey et al., 2017). As gluconeogenesis is an energy-consuming pathway, it is favored when the cell is rich in ATP and the above-named precursors or under glucose-deprived conditions (Berg JM et al., 2002, Michalik et al., 2012). Accordingly, in *E. coli*, the gluconeogenic carbon sources succinate, arabinose and glycerol induce *pckA* expression (Goldie, 1984, Shrago and Shug, 1969). In contrast, the pathway is suppressed by carbon catabolite repression (CCR) when levels of the end product (glucose) are high (Gorke and Stulke, 2008).

The first committed reaction in gluconeogenesis is the decarboxylation and phosphorylation of oxaloacetate to phosphoenolpyruvate (PEP). The reaction is catalyzed by the enzyme phosphoenolpyruvate carboxykinase A (PckA) (Fig. 6) (Scovill et al., 1996). In the gram-positive model organism *B. subtilis*, it has been shown that PckA can also catalyze the reaction from PEP to oxaloacetate at a lower efficiency (Zamboni et al., 2004). Surprisingly for a highly conserved protein like PckA, the oligomerization also shows species-dependent differences. In *E. coli*, PckA is active as a monomer, whereas in *B. subtilis* it was found to be active in di- or tetrameric form (Sauer and Eikmanns, 2005). Currently, no data is available in *S. aureus* regarding the oligomerization of PckA and its ability to carboxylate PEP.

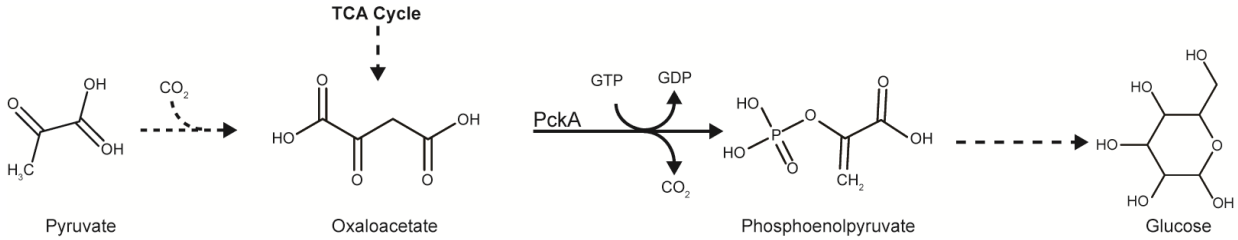

Fig. 6: PckA mediates the initial step of gluconeogenesis

PckA decarboxylates oxaloacetate to PEP. Substrates for the reaction are derived from glycolysis or the TCA cycle. PEP is further processed to glucose.

The expression of *pckA* is regulated by CCR. The promoter region contains a binding site for CcpA (carbon catabolite repression protein A) (Scovill et al., 1996). In presence of glucose, CcpA represses gluconeogenesis by mediating the repression of *pckA* (Seidl et al., 2009). Additionally, high glucose concentrations reduce the expression of important virulence-related traits like *agr, icaADBC, spa* and the superantigen *tst* (Regassa et al., 1992, Seidl et al., 2008, Seidl et al., 2006). PEP fulfills a dual role in the carbohydrate metabolism. As mentioned above, it is an energy rich intermediate in the pathway and can be further metabolized to glucose (Anderson and Wood, 1969). Additionally, it serves as the donor of phosphoryl groups to sugars that get imported by the phosphotransferase system transporters, a system exclusively found in bacteria (Kotrba et al., 2001, Postma et al., 1993). Fructose-6-phosphate is an important intermediate in the gluconeogenesis pathway because it is the starting point for many pathways that are central to *S. aureus* physiology e.g. the synthesis of peptidoglycan and capsule polymers (Sadykov et al., 2010).

I.4 Competition and cooperation in biofilms

Bacteria sharing the same or adjacent physical spaces rarely just passively coexist. More likely, they engage in cooperative or competitive interactions. These interactions are a driving force of evolution because they lead to an arms race in adaptations (Van Valen, 1973). As biofilms have a high local cell density, usually reduced mobility and limited diffusion, intercellular interactions are ubiquitous in biofilms (Teal et al., 2006, Xavier and Foster, 2007). Biofilms are often regarded as the microbial equivalent of a multicellular organisms (Shapiro, 1988). Hence, it was proposed that cooperation would be a prevalent form of interaction in biofilms (Crespi, 2001). This view is supported by findings that mixed-species biofilms can be formed by species that do not form single-species biofilms (Periasamy and Kolenbrander, 2009, Sharma et al., 2005). It was demonstrated that interspecies cooperation in biofilms allows for colonization of niches that would otherwise be inaccessible to the species (Filoche et al., 2004a, Filoche et al., 2004b, Palmer et al., 2001). Cross-feeding (or syntrophy) of nutrients, cofactors or small molecules is another example of true cooperation in biofilms (Harcombe, 2010, Moller et al., 1998, Seth and Taga, 2014, Sztajer et al., 2014). In biofilms, cross-feeding is facilitated by the high cell density and limited diffusion that prevents rapid dilution of the exchanged goods. While cross-feeding conventionally refers to nutrients, the production and exchange of common goods is even more common in biofilms. Cooperative behavior has been described for the production of antibiotics and toxins (Dinges et al., 2000, Riley and Wertz, 2002), immune modulation (Brown, 1999, Hooi et al., 2004) and the production of the extracellular matrix (Davies and Geesey, 1995, Rainey and Rainey, 2003). Despite these evidences for cooperation in biofilms, the view that cooperation is a strict requirement for the development of characteristic structures has been challenged (Claessen et al., 2014). The development of biofilm characteristics has also been interpreted as the cumulative effect of adaptations of individual cells (Klausen et al., 2006, Monds and O'Toole, 2009).

A case study of the production of the extracellular matrix highlights the antagonism between competitive and seemingly cooperative behaviors. The extracellular matrix is a common

good as it also protects non-producers in the colony. However, it can also be used to push matrix producers to the top of the colony towards oxygen and nutrients while suppressing non-producers (Xavier and Foster, 2007, Xavier et al., 2005). The complicated dynamics between cooperation and competition in biofilms have been assessed practically and theoretically (Frank, 2003, Griffin et al., 2004, Nadell et al., 2016, Nadell et al., 2009, Nowak, 2006, West and Buckling, 2003, Xavier and Foster, 2007). Cooperative behavior in microbial communities is at risk of being exploited by cheaters (or non-cooperators) that do not contribute to the production and common goods but profit from their benefits (Czaran and Hoekstra, 2009). Hamilton's theory of kin selection was developed to consolidate this putative discrepancy (Hamilton, 1964). The theory states that the relative benefit of cooperative behavior (b) depends on the factor of relatedness between the actor and beneficiary (r). For genetically identical individuals, the factor r is set to "1". The cooperative behavior is evolutionary stable if the relative benefit is higher than the cost of the behavior (c) (Hamilton, 1964).

$$r * b - c > 0$$

Several mechanisms have been described to ascertain that the actor and the beneficiary are genetically related. In biofilms, dispersal is limited and therefore related cells are often found in close proximity (Nadell et al., 2016, West et al., 2006). Hence, public goods can be shared freely as they are more likely to benefit related cells (Kummerli et al., 2009). The absence of genetic conflicts poses an explanation why cooperation is more common in single-strain biofilms (Xavier and Foster, 2007). On the other hand, high relatedness increases local competition and reduces cooperation on a small scale (Griffin et al., 2004). In kin discrimination, the nature of the public good ensures that it is only of benefit to related cells. The different types of AIP signaling molecules of *S. aureus* are an example of kin discrimination. Non-related cells producing a different AIP type do not benefit from the quorum sensing system; in contrast their quorum sensing system can be cross inhibited by

incompatible AIP signals (Geisinger et al., 2012, Ji et al., 1997, McDowell et al., 2001, Otto et al., 2001).

As mentioned above, cooperation and competition are closely linked interactions on a local scale (Griffin et al., 2004). Exploitative competition mechanisms – competing for the same limited resources – as well as interfering competition mechanisms – actively targeting the competitors – are found in biofilms (Birch, 1957, Rendueles and Ghigo, 2012, Xavier and Foster, 2007). The effects of competition include slowed growth, negative effect on attachment and dispersal of the biofilm (Rendueles and Ghigo, 2012). In contrast to cooperating bacteria which grow co-aggregated in biofilms, competing bacteria often segregate into distinct populations (Elias and Banin, 2012, Nadell et al., 2010).

The interactions of *S. aureus* biofilms with several other microbial species have been studied. As *S. aureus* and *P. aeruginosa* frequently co-colonize the lungs of cystic fibrosis patients, their interaction is of special interest. *P. aeruginosa* produces the small molecule 2-heptyl-4-hydroxyquinoline-N-oxide that triggers σ^B –dependent biofilm formation and the small colony variant (SCV) phenotype in *S. aureus* (Hoffman et al., 2006, Mitchell et al., 2010). The outcome of mixed-species biofilms between *S. aureus* and *P. aeruginosa* is influenced by the environment. Albumin from the human serum interferes with the quorum sensing system of *P. aeruginosa* and shifts the balance in the biofilm towards *S. aureus* (Smith et al., 2017). Nevertheless, there are also reports that *S. aureus* is outcompeted by *P. aeruginosa* or has severely reduced viability in mixed-species biofilms (Biswas et al., 2009, Filkins et al., 2015). The different niches found in biofilms can select for variants and thus change the outcome of an interaction (Boles et al., 2004, Kirisits et al., 2005).

The extracellular matrix of *S. aureus* biofilms is the target in competition with *S. epidermidis* which secretes the protease Esp (Iwase et al., 2010). Esp blocks the release of eDNA to the matrix by interfering with the action of the autolysin Atl (Chen et al., 2013). Established *S. aureus* biofilms can be eradicated by this mechanism. Even though *S. aureus* and *S. epidermidis* are competitors in the human nares, the physiological relevance is reduced by

the fact that not all *S. epidermidis* strains produce Esp (Iwase et al., 2010, Yan et al., 2013). *E. coli* has also been shown to eliminate *S. aureus* from mixed-species biofilms (Makovcova et al., 2017, Millezi et al., 2012). *E. coli* secretes a polysaccharide that prevents adhesion of *S. aureus* and leads to exclusion from the biofilm (Rendueles et al., 2011). Conversely, *S. aureus* can also negatively affect other species in mixed-species biofilms. PSM, which are expressed in an *agr*- and cell density-dependent manner, were shown to possess antimicrobial activity against *Streptococcus pyogenes* (Joo et al., 2011).

It has been observed that carriage of *S.aureus* in the nares can be mono- as well as polyclonal, suggesting the presence of competition mechanisms that are active within the species (Cespedes et al., 2005). As highlighted in chapter I.2.3, competition in a monospecies biofilm can select for mutations in the *sigB* operon causing increased production of bacteriocins and surfactants to outcompete the native strain (Koch et al., 2014b). The type VII secretion system was described as a required factor for persistent infections, abscesses and the modulation of the host immune system (Burts et al., 2008, Burts et al., 2005, Korea et al., 2014). Recently, it was described that the system additionally encodes a toxin-antitoxin-like module that is able to inhibit the growth of *S. aureus*. The module is comprised of a nuclease and a proteinaceous antitoxin (Cao et al., 2016). This intraspecies competition mechanism appears to be prevalent because even strains that do not harbor the toxin were found to carry the antitoxin in their genome (Cao et al., 2016). It is currently not known if this type VII secretion system-dependent competition mechanism is active during biofilm formation of *S. aureus*.

Overall, bacteria in biofilms are engaged in cooperative as well as competitive interactions. To consolidate these contrasting behaviors it was suggested that the view of biofilms as the equivalent of a multicellular organism (O'Toole et al., 2000) should be replaced by the comparison to a multi-organism group, e.g. a flock of birds (Nadell et al., 2009). It was reasoned that biofilms and those group share a characteristic alternating or simultaneous presence of cooperation and competition (Nadell et al., 2009).

I.5 Bacteriophages and prophage induction

Bacteriophages – naturally occurring viruses that specifically target bacterial cells – are estimated to be the most abundant biological entity of the biosphere with approximately 10^{31} virions (Suttle, 2005). Phages are built of a DNA-containing protein capsid with an attached tail of variable length depending on the phage family that holds a base plate and thin tail fibers at the opposite end (Fig. 7). The base plate initiates binding to the bacterial surface (Krzywy et al., 1981, Spilman et al., 2011, Xia and Wolz, 2014, Yap and Rossmann, 2014). Bacteriophages were named after their ability to destroy bacterial cells (greek *phagein* = "to eat"), but this is only one of their possible lifestyles (D'Herelle, 1917, Twort, 1915). Next to the lytic lifestyle, bacteriophages use a process termed lysogeny to stably but reversibly integrate into the bacterial chromosome forming a prophage (Blair and Carr, 1961, Lwoff and Gutmann, 1950). Prophages are quiescent phages that give the lysogenic strain resistance against infection with that particular phage (Geli and Corda, 1998). Dormancy of prophages is controlled by repressors that silence genes of the lytic cycle (Biswas et al., 2017, Das et al., 2007, Waldor and Friedman, 2005). The factors that control the decision between the lytic and lysogenic cycle upon phage infections are largely obscure. Only recently, a quorum sensing-like system of phages that influences the decision based on phage-abundance has been described in *B. subtilis* (Erez et al., 2017). Prophages can enter the lytic cycle again, produce phage particles and lyse their host cell.

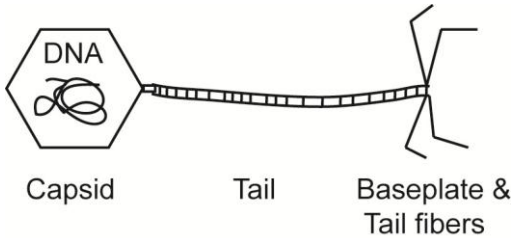

Fig. 7: Schematic representation of a *Siphoviridae* bacteriophage

Siphoviridae are the best-characterized phage family in *S. aureus*. The capsid holds 40-50 kb DNA and is connected to the baseplate and tail fibers by a non-contractile tail. Adapted from (Elbreki et al., 2014) published under CC BY 3.0.

The induction of prophages can occur spontaneously but most commonly occurs due to activation of the SOS response of the host cell (Goerke et al., 2006, Maiques et al., 2006,

Nanda et al., 2015, Selva et al., 2009). The SOS response is a bacterial global stress response system triggered by DNA damage that activates genes associated with DNA repair (Courcelle et al., 2001, d'Ari, 1985, Fernandez De Henestrosa et al., 2000). The protein RecA filaments upon sensing of single-stranded DNA and switches to an active confirmation. This activates autocleavage of the transcriptional repressor LexA and SOS response genes as well as genes related to the lytic cycle of bacteriophages get expressed (Butala et al., 2009, Fernandez De Henestrosa et al., 2000). Factors that trigger the SOS response in staphylococci include UV radiation and the antibiotics ciprofloxacin, ampicillin, penicillin, mitomycin C and trimethoprim (Cirz et al., 2007, Courcelle et al., 2001, Goerke et al., 2006, Maiques et al., 2006, Silva and Leitato, 1984, Vestergaard et al., 2015).

Bacteriophages have a narrow spectrum of bacterial hosts that they can infect. Therefore, phage typing can be used as a tool to characterize *S. aureus* strains (Kali et al., 2013, Pantucek et al., 2004, Wentworth, 1963). Adsorption of phages to the bacterial cells is the first step of the infection process and determines specificity (Braun and Hantke, 1977). Extracellular components like the cell wall and wall-teichoic acids are receptors for phages (Chatterjee, 1969, Shaw and Chatterjee, 1971, Xia et al., 2011). In contrast, SpA masks these receptors and increased production of the proteins negatively affects adsorption effectivity (Nordstrom and Forsgren, 1974). Additionally, phage-related factors i.e. proteins on the base plate, are required for adsorption to the bacterial cell (Li et al., 2016). After adsorption, the phage DNA is translocated across the cell membrane to complete infection, leaving an empty protein capsid (Grayson and Molineux, 2007, Xu and Xiang, 2017). At this point, phages either enter lysogeny or the lytic cycle in which the phage DNA is strongly transcribed. The produced phage particles are empty at first and later packaged with the phage genome. This process generates the internal pressure that can be used to translocate the DNA across the host membrane during infection (Rickgauer et al., 2008, Smith et al., 2001, Xu and Xiang, 2017). The phage particles are packed with up to 45 kb of DNA, equivalent to 1.5% of the staphylococcal genome (Morse, 1959, Ubelaker and Rosenblum, 1978). To be released from the host cell, the membrane and cell wall are attacked

enzymatically by holins and endolysins, respectively, until the cell bursts open and releases the phage particles into the environment (Loessner et al., 1999, Young, 1992, Young and Blasi, 1995).

The ecological impact of phages is not only exercised through lysis of cells or communities but also by their role in horizontal gene transfer (HGT) (Brussow et al., 2004, Bushman, 2001, Moon et al., 2016, Quiles-Puchalt et al., 2014). Mobile genetic elements make up 15-20% of the staphylococcal genome and bacteriophages have been implicated with the transfer of these elements (Lindsay and Holden, 2004, Malachowa and DeLeo, 2010, Moon et al., 2016). Pathogenicity islands are small genomic areas containing virulence genes that can be transferred horizontally with the aid of helper phages (Lindsay et al., 1998, Tallent et al., 2007, Tormo et al., 2008). The islands are highly dynamic and evidence for the exchange of genetic material between different islands has been reported (Subedi et al., 2007). In addition to mobilizing virulence factors, bacteriophages can also mobilize antibiotic resistance genes accelerating the spread of antibiotic resistance through bacterial communities (Colomer-Lluch et al., 2011, Haaber et al., 2016).

I.6 Objectives of this work

The macrocolony biofilm model is used to study processes related to the architecture of multicellular bacterial communities as well as differentiation and heterogeneity (Serra and Hengge, 2014). While the genetic pathway that controls macrocolony aggregation in *S. aureus* has been studied, little is known about accessory factors involved and the metabolic requirements (Garcia-Betancur et al., 2017). The first part of the thesis attends to the question which genetic elements influence the morphology of macrocolonies by screening a genome-wide transposon library (Fey et al., 2013). Newly identified regulators will be characterized to determine how they contribute to the establishment of the macrocolony morphology. These findings will improve our understanding of a novel *in vitro* model of biofilm formation.

The second part of this thesis addresses the processes occurring when two strains of the same species compete in the same ecological niche, e.g. a macrocolony biofilm. Previous experimental approaches using the macrocolony biofilms have shown that the model accurately reflects community-wide effects that similarly occur *in vivo* during infections (Garcia-Betancur et al., 2017, Koch et al., 2014b). The nature and outcome of social interactions is highly dynamic and alterable by environmental factors *in vivo*. The necessity to study social interactions has recently been highlighted by the appreciation of competition strategies between *S. aureus* and closely related species sharing an ecological niche as a potential source of future antimicrobials (Paharik et al., 2017, Zipperer et al., 2016).

II. Results

II.1 Identification of genes involved in macrocolony formation in *S. aureus*

II.1.1 Macrocolony aggregation is induced by magnesium

It was recently unraveled that the aggregation of *S. aureus* into macrocolony biofilms in magnesium-supplemented TSB medium (TSBMg) relies on the σ^B-dependent repression of Agr (Garcia-Betancur et al., 2017). This section of the thesis aims to generate a deeper understanding of the mechanisms of macrocolony biofilm formation and the genes involved in the development of the biofilm architecture.

To study the specificity of magnesium-dependence of macrocolony biofilm formation, TSB agar was supplemented individually with several salts ($CaCl_2$, $MgCl_2$, $MgSO_4$, KCl, $MnCl_2$, NaCl). It was tested if the salts induced aggregation of *S. aureus* into macrocolonies on agar plates and the formation of submerged biofilms in liquid medium. In addition, the conventional biofilm medium TSB supplemented with 3% NaCl and 0.5% glucose (TSB-NaCl-glu) was assessed for its effect on the formation of macrocolony and submerged biofilms (Beenken et al., 2004). Multicellular aggregates formed in TSBMg and TSB with 100 mM $MgSO_4$, albeit to a weaker extent, on solid agar as well as in the submerged biofilm formation assay. Multicellular aggregation and formation of submerged biofilms was not found in TSB with other supplements ($CaCl_2$, KCl, $MnCl_2$, NaCl, NaCl + glu) (Fig. 8a-c), which emphasizes the requirement for Mg^{2+} in the medium to trigger multicellular aggregation. Growth curves of *S. aureus* cultured in growth medium with $MgCl_2$- or $MgSO_4$-supplement were similar, hence a relation of differences in biofilm formation with growth alterations was ruled out (Fig. 8d).

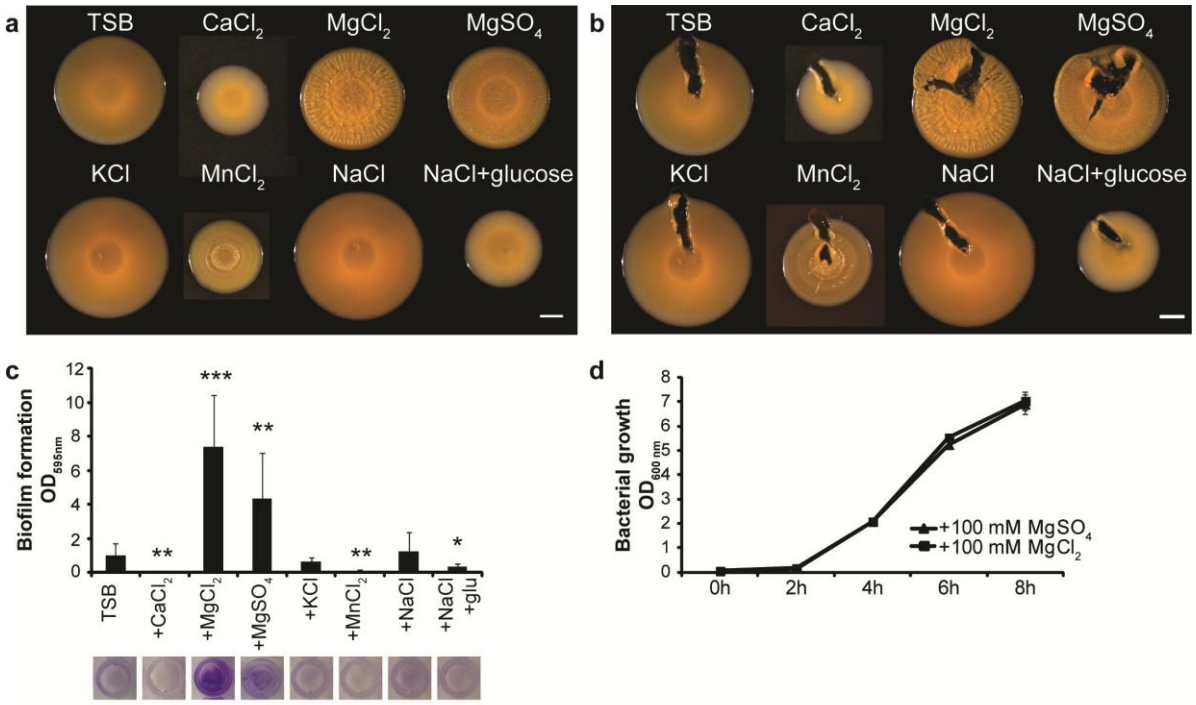

Fig. 8: Macrocolony biofilm formation in *S. aureus* depends on magnesium.

(**a**) Macrocolonies of strain Newman grown on TSB with different supplements. Distinctive macrocolony wrinkling is only induced by magnesium salts. (**b**) Resistance to macrocolony dispersal is only induced by magnesium salts. (**c)** Only magnesium salts induce biofilm formation in a quantitative biofilm assay. (**d**) Anion in the magnesium salt added to culture medium (TSB + 100 mM $MgCl_2$ or 100 mM $MgSO_4$) does not affect growth of strain Newman. Macrocolonies were imaged after 5 days. Scale bars, 2mm. Graphs in (**c**,**d**) show mean ± SD of 3 independent experiments. Statistical significance was calculated using unpaired Student's t-test using unsupplemented medium as reference. *p ≤0.05, ** p ≤0.01, *** p ≤0.001

Structural elements, e.g. PNAG and SpA, are required for biofilm formation in addition to the activity of regulators including Agr and σ^B (Clarke and Foster, 2006, Cramton et al., 1999, Heilmann, 2011, Lauderdale et al., 2009, Merino et al., 2009). The macrocolony morphology of mutants for the respective genes was assessed to determine the role of these factors in macrocolony aggregation formation. Agr-deficiency increased robustness and wrinkling of the macrocolony, whereas the deletion of σ^B reduced macrocolony complexity and wrinkling. The macrocolony morphology of a strain deficient in PNAG and SpA was more fragile with reduction of wrinkling (Fig. 9). A positive correlation between the wrinkling of the macrocolony aggregate and the biofilm strength determined in conventional assays with liquid medium was found. The biofilm regulators identified in submerged biofilms generate

similar effects on macrocolony biofilms. Therefore, macrocolonies were considered a suitable model for studies of biofilm-related processes in *S. aureus*.

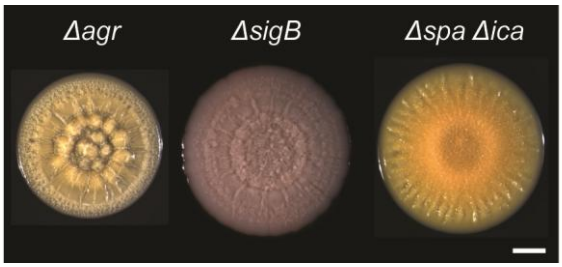

Fig. 9: Biofilm regulators affect macrocolony morphology.

The macrocolony morphology of the strain Newman on TSBMg is affected by the absence of the genes *agr*, *sigB*, *ica* and *spa* according to their roles in submerged biofilms. Macrocolonies were imaged after 5 days. Scale bar, 2 mm.

II.1.2 Screening for novel biofilm regulators

The macrocolony biofilm developmental assay was used to search for genes with a role in biofilm formation that were undetected in previous studies using the submerged biofilm formation assay. The macroscopic macrocolony architecture of the strains of a genome-wide transposon-mapped collection of *S. aureus* mutants (1920 mutants) (Nebraska transposon mutant library, NTML) was evaluated to identify new regulators (Fey et al., 2013). The parent strain of the library, USA300-JE2, forms robust, structured macrocolonies on TSBMg (Fig. 10a). Macrocolonies of the strain were seeded on TSBMg agar plates by two approaches: (i) 2 µl of a dense suspension of cells was spotted onto the plates or (ii) cellular material was directly transferred from TSB plates. Comparison of the colony morphologies showed that macrocolonies spotted from suspension had a more uniform shape due to the more homogenous distribution of cells, but overall, both methods lead to similar macrocolony morphologies (Fig. 10b). To screen for novel biofilm regulators, strains of the NTML were grown with an intermediate incubation step on TSB agar for 24 h before incubation on TSBMg agar for up to 120 h (Fig. 10c).

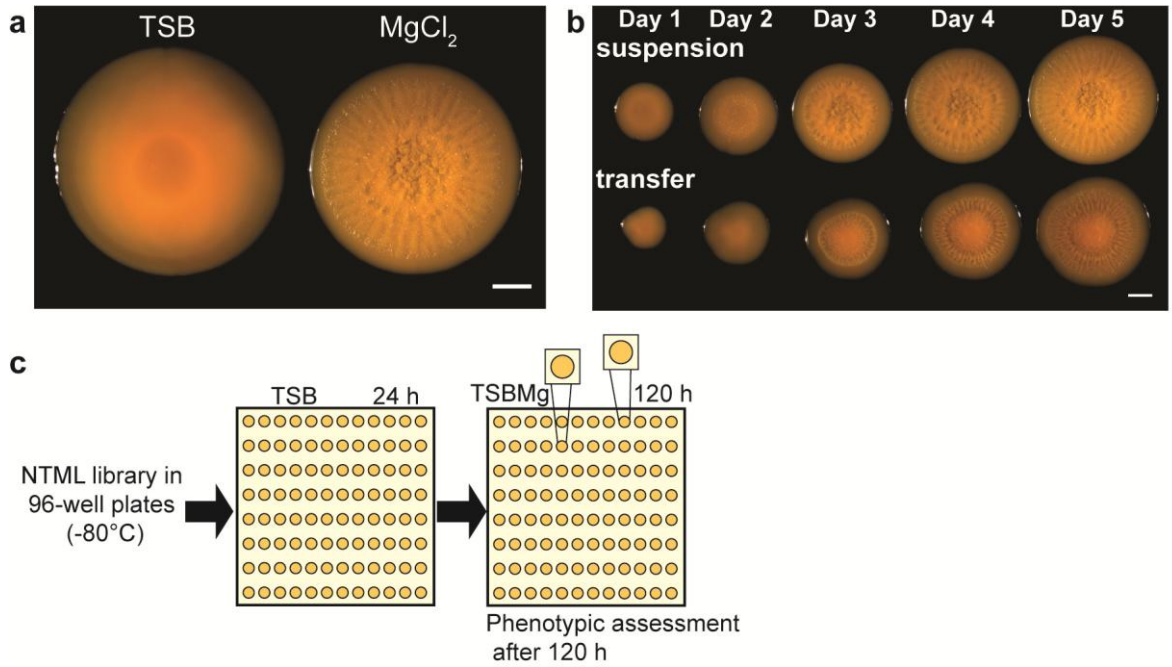

Fig. 10: USA300-JE2 forms macrocolony biofilms.

(**a**) Macrocolony formation of USA300-JE2 only occurs on TSBMg but not on TSB. Macrocolonies were imaged after 5 days. (**b**) Progression of USA300-JE2 macrocolonies over 5 days. Comparable development of the macrocolonies is observed when they are spotted from cell suspension or by transfer of cellular material. (**c**) Scheme of the NTML screen to identify modulators of macrocolony morphology. Scale bars, 2 mm.

In the screen, 28 transposon mutants with affected genes that were not previously associated with biofilm formation were identified (Fig. 11c-e and Table 1).The transposon insertions in the 28 genes that showed altered macrocolony phenotypes were validated by PCR-amplifying and subsequently sequencing the chromosome/transposon junctions (Fig. 12). The results confirmed the transposon insertions as they were annotated in the NTML (Fey et al., 2013). Based on the colony surface structure and wrinkling, which is related to the production of ECM (Branda et al., 2006, Ray et al., 2012), these macrocolonies were visually inspected and grouped into three categories: The mutants in category 1 were characterized by a reduction or complete absence of wrinkles at the macrocolony surface (Fig. 11a and c). The two strains that were classified as category 2 also have a macrocolony surface lacking wrinkles. In addition, these strains have a flattened macrocolony phenotype that contrasts the voluminous macrocolonies formed by the WT and the strains in the other categories (Fig. 11a and d). The mutants in category 3 showed a hyperwrinkled macrocolony

surface compared to the WT (Fig. 11a and e). Based on the above-defined characteristics, mutants of the regulators σ^B and Agr were classified into category 1 and 3, respectively (Fig. 11b). Additional phenotypic parameters, i.e. colony diameter and pigmentation, were not considered in the classification despite varying between the WT and the 28 candidates, because they did not correlate with surface wrinkling (Fig. 13).

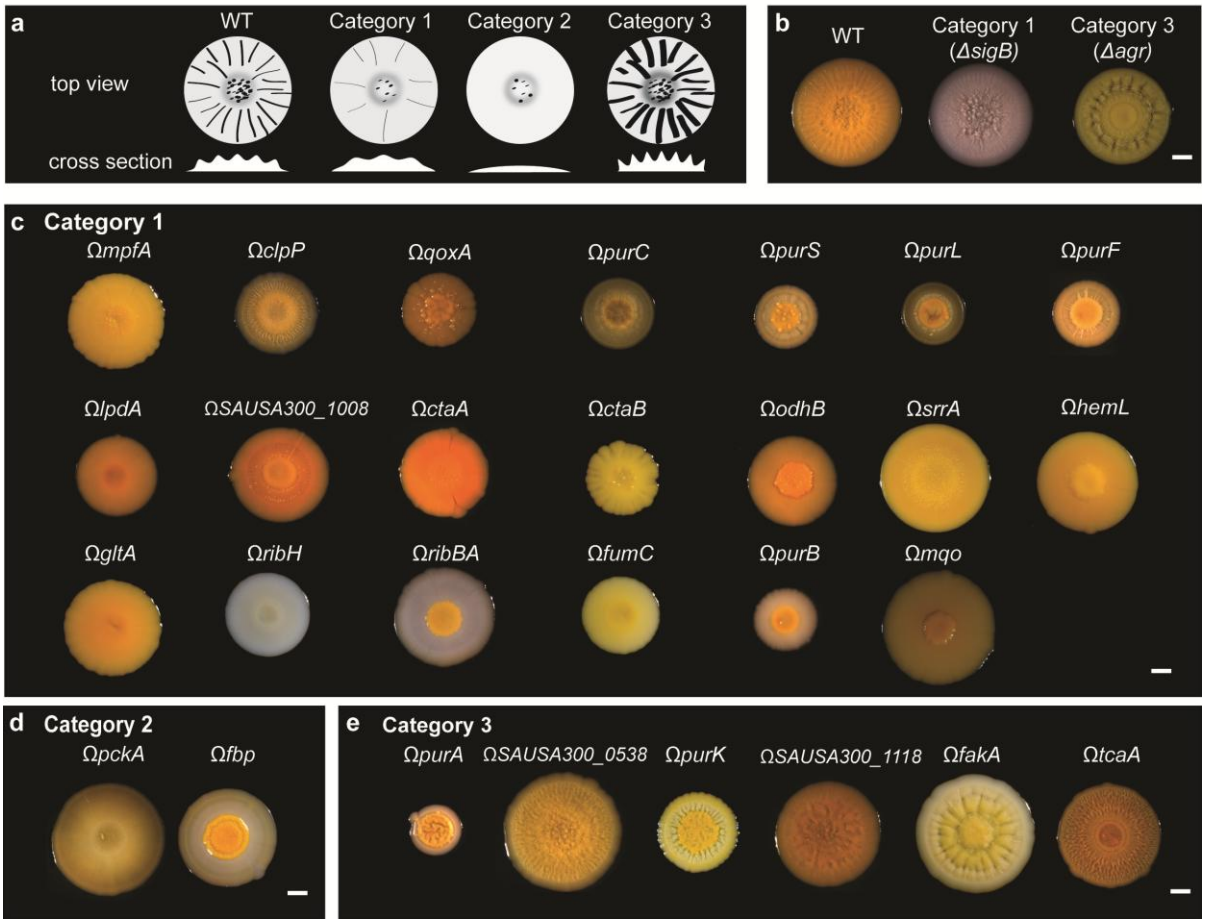

Fig. 11: Identification of novel macrocolony morphology phenotypes in NTML.

(a) Schematic representation of the macrocolony morphology of USA300-JE2 WT and the three newly identified categories. (**b**) WT macrocolony; categories 1 and 3 represented by macrocolonies of mutants of biofilm regulators σ^B and Agr. (**c**) Identified candidates of category 1 display little or no surface wrinkling. (**d**) Identified candidates of category 2 form thin macrocolonies with few or no wrinkles. (**e**) Identified candidates of category 3 have more pronounced wrinkling of the surface. Macrocolonies were imaged after 5 days. Scale bars, 2 mm.

Accession Number	Name	Gene Function	Biological Function	Localization	Phenotypic Category
SAUSA300_0017	purA	adenylosuccinate synthase	*de novo* purine synthesis	cytoplasm	3
SAUSA300_0538	-	UDP-glucose 4-epimerase	unknown	cytoplasm	3
SAUSA300_0687	mpfA	HlyC/CorC family transporter	membrane transporter	membrane	1
SAUSA300_0752	clpP	ATP-dependent Clp protease proteolytic subunit	protein degradation	cytoplasm	1
SAUSA300_0963	qoxA	quinol oxidase	electron transport chain	membrane	1
SAUSA300_0967	purK	5-(carboxyamino) imidazole ribonucleotide synthase	*de novo* purine synthesis	cytoplasm	3
SAUSA300_0968	purC	phosphoribosylamino-imidazolesuccino-carboxamide synthase	*de novo* purine synthesis	cytoplasm	1
SAUSA300_0969	purS	phosphoribosylformyl-glycinamidine synthase	*de novo* purine synthesis	cytoplasm	1
SAUSA300_0971	purL	phosphoribosylformyl-glycinamidine synthase II	*de novo* purine synthesis	cytoplasm	1
SAUSA300_0972	purF	amidophosphoribosyl-transferase	*de novo* purine synthesis	cytoplasm	1
SAUSA300_0996	lpdA	dihydrolipoyl dehydrogenase	redox homeostasis	cytoplasm	1
SAUSA300_1008	-	hypothetical protein	unknown	membrane	1
SAUSA300_1015	ctaA	heme A synthase	cofactor synthesis	membrane	1
SAUSA300_1016	ctaB	protoheme IX farnesyltransferase	cofactor synthesis	membrane	1
SAUSA300_1118	-	Asp23/Gls24 family envelope stress response protein	unknown	cytoplasm	3
SAUSA300_1119	fakA	glycerone kinase	fatty acid synthesis	cytoplasm	3
SAUSA300_1305	odhB	dihydrolipoamide succinyltransferase	TCA cycle	cytoplasm	1

Accession Number	Name	Gene Function	Biological Function	Localization	Phenotypic Category
SAUSA300_1442	srrA	DNA-binding response regulator	oxygen sensing	cytoplasm	1
SAUSA300_1614	hemL	glutamate-1-semialdehyde-2,1-aminomutase	cofactor synthesis	cytoplasm	1
SAUSA300_1641	gltA	citrate synthase	TCA cycle	cytoplasm	1
SAUSA300_1712	ribH	6,7-dimethyl-8-ribityllumazine synthase	coenzyme synthesis	cytoplasm	1
SAUSA300_1713	ribBA	GTP-cyclohydrolase II	coenzyme synthesis	cytoplasm	1
SAUSA300_1731	pckA	phosphoenolpyruvate carboxykinase	gluconeogenesis	cytoplasm	2
SAUSA300_1801	fumC	class II fumarate hydratase	TCA cycle	cytoplasm	1
SAUSA300_1889	purB	adenylosuccinate lyase	*de novo* purine synthesis	cytoplasm	1
SAUSA300_2302	tcaA	zinc ribbon domain-containing protein	antibiotic response	membrane	3
SAUSA300_2312	mqo	malate-quinone oxidoreductase	TCA cycle	cytoplasm	1
SAUSA300_2455	fbp	fructose 1,6-bisphosphatase	gluconeogenesis	cytoplasm	2

Table 1: Candidates identified in the screen of the NTML

In the screen of the NTML, 28 candidates with altered macrocolony morphology were identified. Information on gene function, biological function and subcellular localization was extracted from https://www.uniprot.org. Candidates are ordered by the accession number of the gene in which the transposon is inserted.

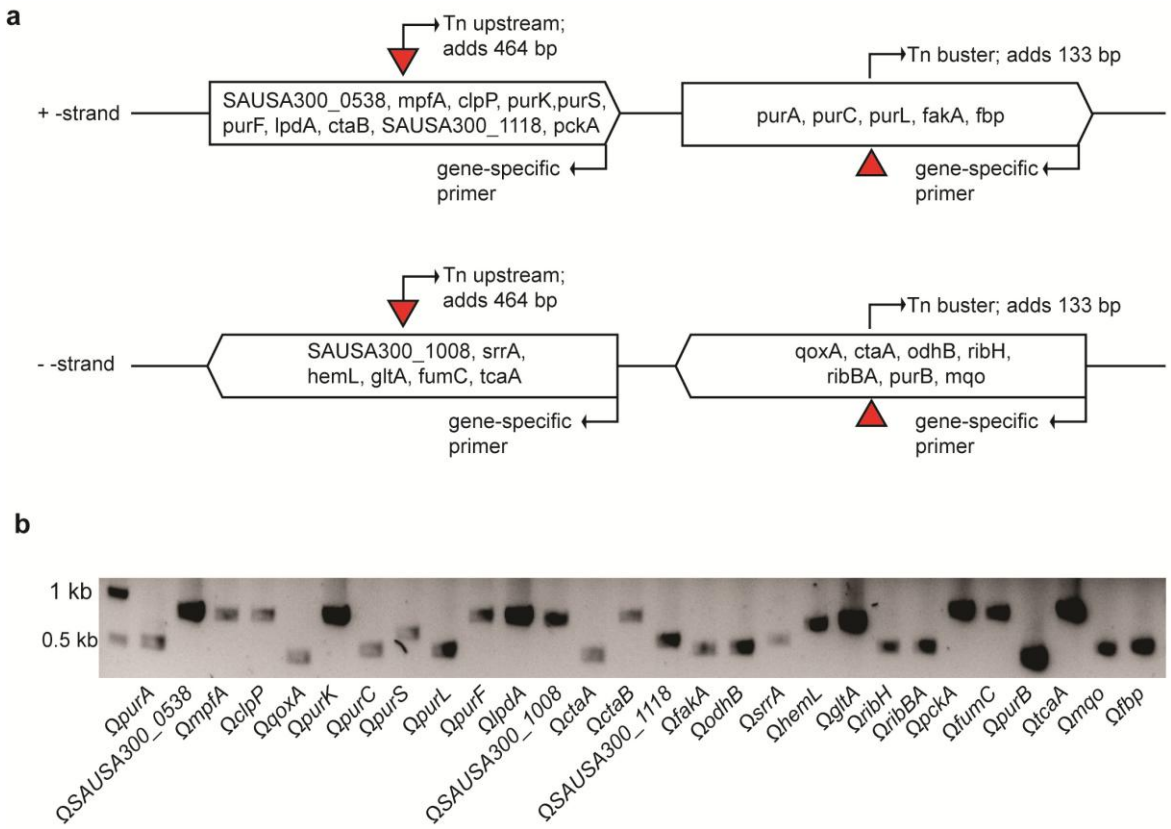

Fig. 12: Sequencing of chromosome/Tn junctions confirms annotated Tn insertions.

(a) Schematic representation of the orientation of the 28 candidate genes and the corresponding transposons (Tn) on the chromosome. The red triangles above and below the gene indicate the transposon in "plus"-orientation and "minus"-orientation, respectively. Information on transposon orientation and transposon-specific primers were retrieved from **http://app1.unmc.edu/fgx/** (Fey et al., 2013). Tn- and gene-specific primers with expected PCR fragment sizes are listed in section **VII.3**. **(b)** PCR products of the chromosome/Tn junction with sizes between 400 and 900 bp. PCR Products were sequenced and aligned to the respective genes to confirm the annotated transposon insertion sites.

The affected genes in the 28 candidates code for cytoplasmatic proteins (79%) and membrane proteins (21%) (Table 1 and Fig. 14a). Analysis of the biological functions of the candidates showed that mutants affected in purine biosynthesis (25%) and carbohydrate metabolism (21%) were the most prevalent, followed by mutants affected in cofactor and coenzyme biosynthesis (18%). Further biological functions found in the screen but not represented by more than two candidates included protein degradation, membrane transport, electron transport chain, redox homeostasis, oxygen sensing, fatty acid synthesis and antibiotic response. These functions were clustered as "other" and represent 25% of

candidates. Finally, for 11% of the candidates, no known function has been assigned to the gene (Table 1 and Fig. 14b).

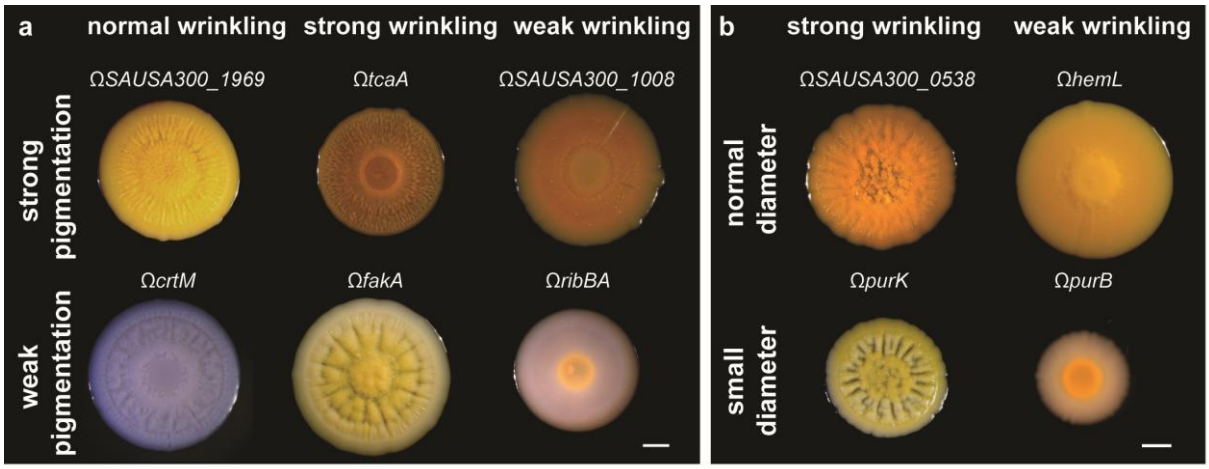

Fig. 13: Macrocolony pigmentation and diameter are not linked to surface wrinkling.

(a) Macrocolony pigmentation is no predictor for surface wrinkling. Regular, increased and decreased wrinkling of the macrocolony surface can be developed by strains showing strong or reduced pigmentation (top and bottom row, respectively) **(b)** Macrocolony diameter is no predictor for surface wrinkling. Strains forming colonies with a regular diameter (top row) can have increased and reduced wrinkling. This variation is also observed in strains forming macrocolonies with a small diameter (bottom row). Macrocolonies were imaged after 5 days. Scale bar, 2 mm.

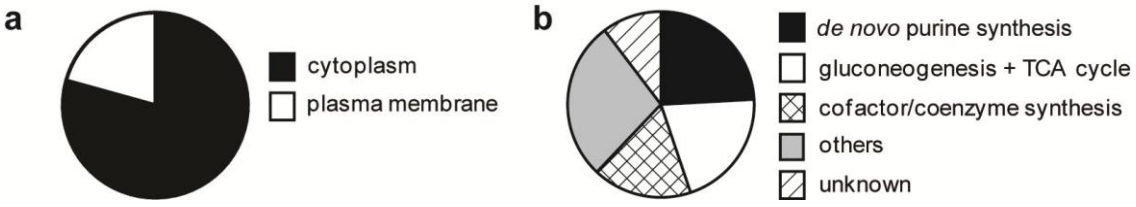

Fig. 14: Classification of NTML candidates.

(**a**) Subcellular distribution of the candidate proteins in the cytoplasm and plasma membrane. (**b**) Biological functions of the candidate proteins.

To assess a potential link between alterations in biofilm formation and growth, cultures of the 28 candidates were grown in liquid medium and their optical density was measured after 10 h. Sixteen of the 28 candidates had a growth defect relative to the WT at this time point (Fig. 15). The threshold for growth deficiency was set at 80% of the optical density measured in the WT at this time point (Wang et al., 2017). Candidates displaying a growth deficiency were excluded from further characterization with the exception of *ΩpurK* because its hyperwrinkled macrocolony phenotype suggested strong biofilm formation despite having

reduced biomass. In the selected mutants, the formation of submerged biofilms was quantified in a crystal violet (CV) assay. The assay was performed in TSBMg and conventional TSB-NaCl-glu biofilm growth medium. Two candidates showed statistically significant alterations of biofilm formation in TSB-NaCl-glu: *ΩclpP* formed more biofilm and *ΩpurK* formed less biofilm than the WT in this growth medium (Fig. 16). In TSBMg, statistically increased biofilm formation was measured in *ΩclpP* and *ΩtcaA* (Fig. 16). Hence, in both biofilm-inducing growth media used in this assay, 11 out of 13 candidates identified in the macrocolony screen did not differ from WT in the formation of submerged biofilms. Use of the macrocolony biofilm assay enables the identification of biofilm-related processes changing the biofilm morphology that can not be detected in the conventional submerged biofilm assay.

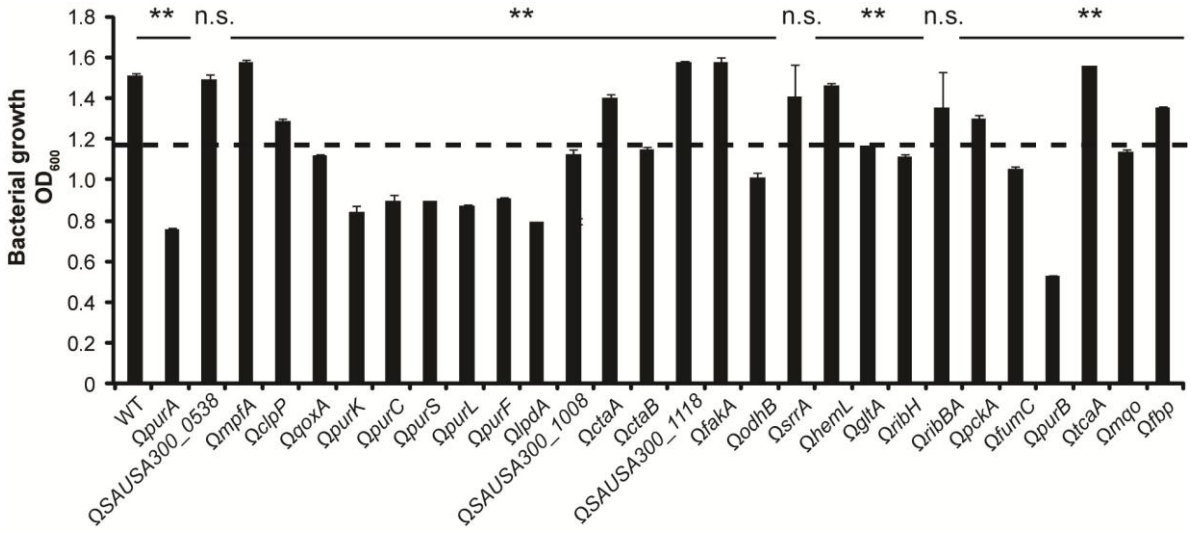

Fig. 15: Growth behavior of NTML candidates.

OD_{600} after growth for 10 h in TSB. Dashed line indicates 80% of the WT OD_{600}. P values were calculated with WT as reference using unpaired Student's t-test. Graphs show mean ± SD of 3 independent experiments. ** p≤0.01, n.s. not significant.

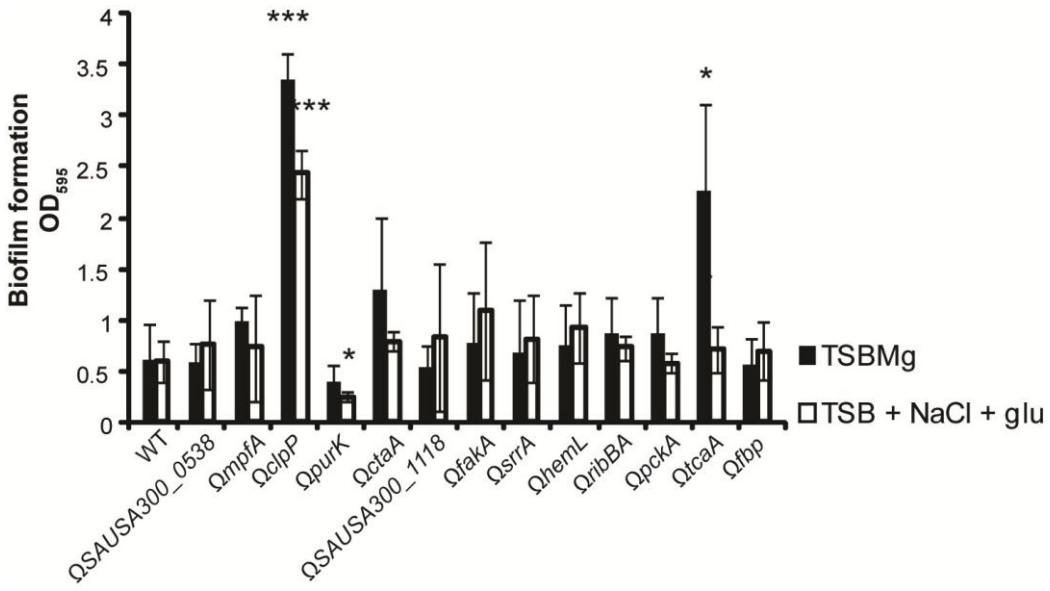

Fig. 16: Biofilm formation of NTML candidates without a growth deficit.

Quantitative assay of submerged biofilms in TSBMg and TSB-NaCl-glu of the non growth-deficient candidates from Fig. 15. P values were calculated with WT as reference using unpaired Student's t-test. Graphs show mean ± SD of 3 independent experiments. * p≤0.05, *** p≤0.001, n.s. not significant.

The structuring of the WT macrocolony surface, which determines the eventual biofilm morphology, occurs between days 3 and 5 (Fig. 10b). The macrocolony progression of the 13 candidates used in the CV assay in Fig. 16 was followed for this period. The mutants classified in the categories 1 and 2 were distinguishable from WT by day 3 due to the absence of surface wrinkling (Fig. 17). In contrast, the increased wrinkling of *ΩpurK* (category 3) only developed after day 4 (Fig. 17). The macrocolonies of *ΩSAUSA300_0538*, *ΩSAUSA300_1118*, *ΩfakA* and *ΩtcaA* (all category 3) have accelerated development as they are clearly structured as early as day 3 (Fig. 17).

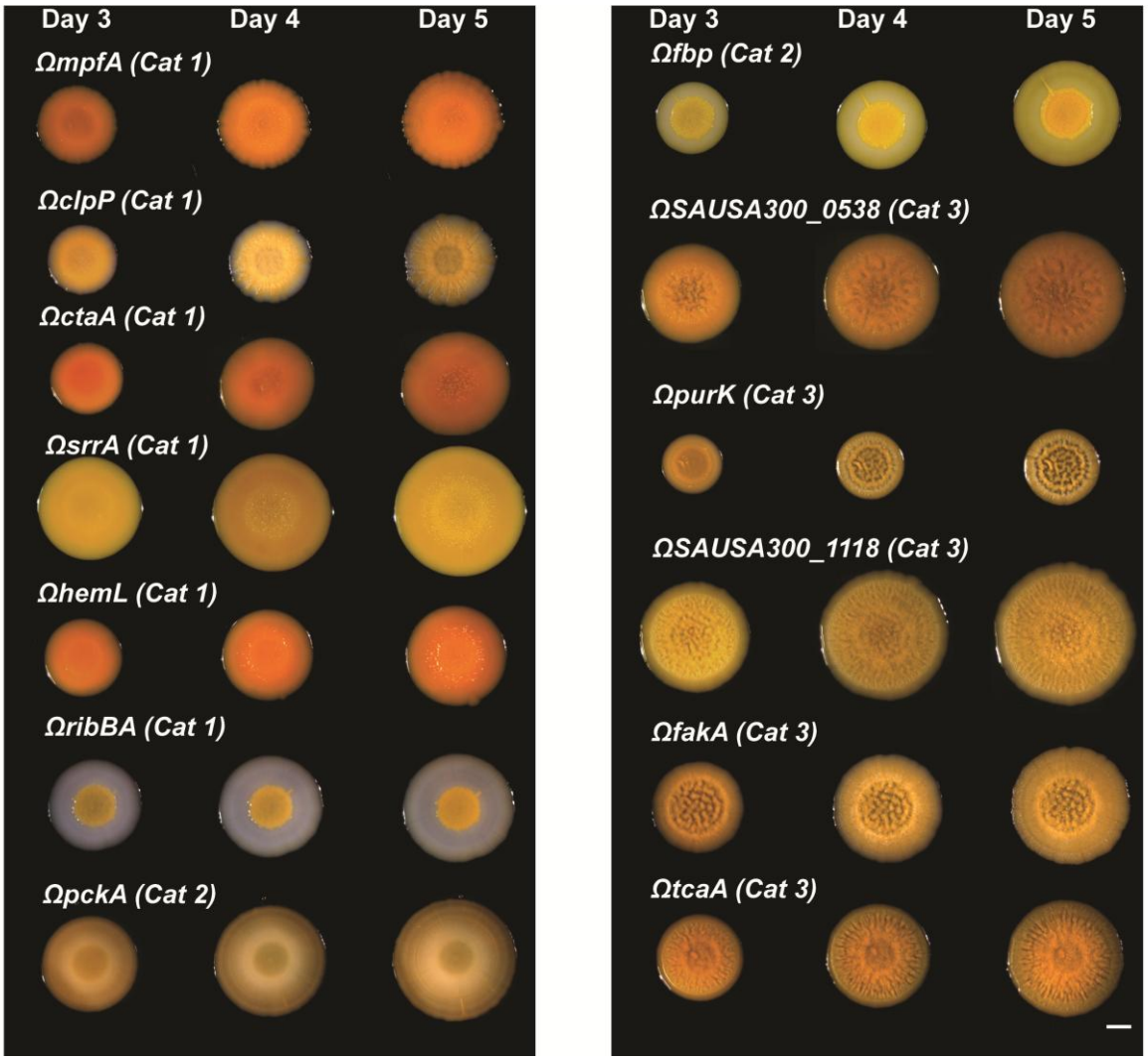

Fig. 17: Macrocolony morphology of candidates after 3 – 5 days.

Category 1 (left), macrocolony morphology differs from WT after 3 days due to lack of wrinkling. Category 2 (bottom left and top right), thin flat morphology is apparent after 3 days. Category 3 (right), accelerated development with clearly structured macrocolonies after 3 days; *ΩpurK* develops wrinkled surface after 4 days. Scale bar, 2 mm.

The transposon mutants *ΩsrrA*, *ΩpckA* and *ΩpurK* were chosen for further characterization of the macrocolony phenotypes with additional experiments. These strains cover the three phenotypic categories (Fig. 11a) and represent a broad range of the macrocolony morphologies identified in the NTML screen. Moreover, *purK* and *pckA* have gene functions related to purine biosynthesis and carbohydrate metabolism, respectively, which were the most abundant functional categories found in the screen. Mapping of the NTML to determine the exact insertion sites of the transposon in all the strains is available (Fey et al., 2013). The transposon insertions in *pckA*, *purK* and *srrA* are located in the first 30% of the ORF making

residual gene functions unlikely. Nevertheless, deletion mutants in a plasmid-bearing multidrug-resistant clinical isolate of the USA300 lineage (USA300_TCH1516) were generated (Gonzalez et al., 2005). The macrocolonies of these deletion strains display the same macrocolony morphology found in the transposon mutants in USA300-JE2 and were used for all subsequent experiments.

II.1.3 Purine biosynthesis affects macrocolony formation

De novo biosynthesis of purines, which is required for virulence and rifampicin resistance in *S. aureus*, was the most abundant functional category among the 28 candidates identified in the screen (Lan et al., 2010, Yee et al., 2011). The process is mediated by an 11-gene operon, *purEKCSQLFMNHD*, two additional genes, *purA* and *purB,* and the repressor *purR (Baxter-Gabbard and Pattee, 1970)* (Fig. 18). Of these 14 genes, 5 transposon mutants had a less wrinkled macrocolony morphology (*ΩpurB, ΩpurC, ΩpurF, ΩpurL* and *ΩpurS*), two mutants had stronger wrinkling (*ΩpurA* and *ΩpurK*), 6 mutants did not show a distinct phenotype (*ΩpurD, ΩpurH, ΩpurM, ΩpurN, ΩpurQ* and *ΩpurR*) and one gene was not found in the NTML (*purE*) (Fig. 11). It was not possible to generate a strain carrying a transposon insertion in *purE*; hence the gene is potentially essential (Fey et al., 2013).

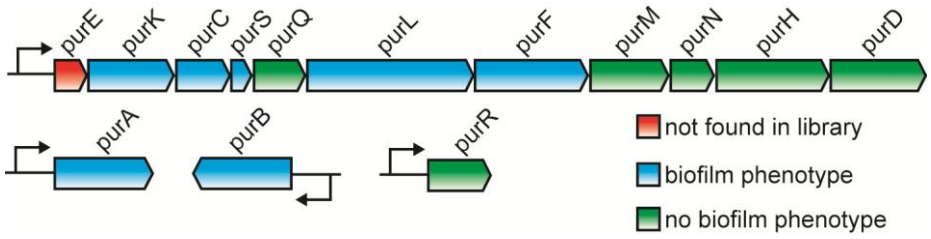

Fig. 18: Organization of genes in purine biosynthesis

The purine synthesis-related genes are organized into a large operon and three monocistronic genes. All macrocolonies with a distinct phenotype are shown in Fig. 11.

The gene *purK* was chosen as a case study for the role of purine biosynthesis in biofilm formation in *S. aureus*. The macrocolonies of *ΔpurK* had a smaller diameter than the WT, which is a common feature for *pur*-deficient candidates identified in the screen, and the core displayed pronounced wrinkles (Fig. 19a). The aggregates had a rigid structure and fragmented into small pieces upon dispersal with a blunt object (Fig. 19a).

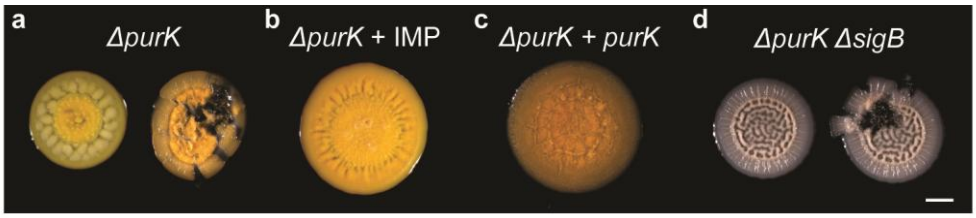

Fig. 19: Macrocolony phenotype of a purine synthesis-defective strain

(**a**) Macrocolonies of *ΔpurK* have smaller diameter than WT macrocolonies, pronounced wrinkling (left) and resist dispersal with blunt objects (right). (**b**) Metabolic complementation of the *ΔpurK* phenotype by IMP (**c**) Complementation of *ΔpurK* phenotype by *purK* expression. (**d**) *ΔpurK ΔsigB* macrocolony overproduces wrinkles and breaks into fragments upon dispersal. Macrocolonies were imaged after 5 days. Scale bar, 2 mm.

Inosine monophosphate (IMP) is the last common intermediate of adenine and guanine biosynthesis. IMP biosynthesis occurs downstream of the enzymatic activity of PurK (Hartman and Buchanan, 1959). To assess if IMP can metabolically complement the purine deficiency of *ΔpurK*, IMP solution was spotted on the agar, briefly dried and macrocolonies were seeded on top. IMP-supplemented macrocolonies showed an increased colony diameter and reduced wrinkling compared to the untreated macrocolonies (Fig. 19b). Expression of *purK* from a neutral locus restored WT-like levels of wrinkling and colony diameter in *ΔpurK* macrocolonies (Fig. 19c). To determine if σ^B, the major positive regulator of macrocolony aggregation, was involved in the *ΔpurK* phenotype, a *ΔpurK ΔsigB* mutant was generated. This double mutant is non-pigmented, which is characteristic for *ΔsigB* strains (Nicholas et al., 1999), and maintains the *ΔpurK* phenotype, i.e. a hyperwrinkled macrocolony with a small diameter (Fig. 19d). Therefore, the *ΔpurK* macrocolony phenotype is not dependent on the activation of σ^B.

The growth defect of *ΔpurK* could reduce the biomass, which is produced in biofilms of the strain. The crystal violet assay of submerged biofilms quantifies the total biofilm-bound biomass regardless of biofilm architecture (Merritt et al., 2005). Therefore, the biofilm formation of *ΔpurK* and WT was quantified using this assay in different growth media (TSB, TSB-NaCl-glu, TSBMg) to compare total biofilm formation. In TSB and TSB-NaCl-glu, *ΔpurK* and WT did not differ in biofilm formation but both strains showed increased biofilm formation in TSBMg. The increase in *ΔpurK* was more pronounced leading to biofilm formation levels

twice as high as in the WT (Fig. 20). This phenotype in the *ΔpurK* deletion mutant in USA300_TCH1516 contrasts the phenotype of the transposon mutant *ΩpurK* in USA300-JE2, which formed less biofilm in TSB-NaCl-glu and did not differ from the WT in TSBMg (Fig. 16). The crystal violet assays in Fig. 16 and Fig. 20 differ in the well volume of the multiwell plate, as 96- and 24-well plates, respectively, were chosen for the assay. It is hypothesized that these variations in the experimental set-up along with the different strain backgrounds and means of gene inactivation were causative for the diverging results.

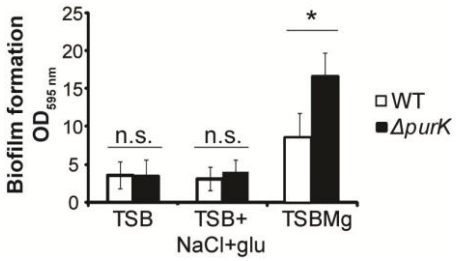

Fig. 20: Quantification of submerged biofilm formation in *ΔpurK*

Biofilm overproduction in *ΔpurK* is dependent on TSBMg and not observed in other growth media. P values were calculated using unpaired Student's t-test. Graphs show mean ± SD of 3 independent experiments. * p≤0.05, n.s. not significant.

Overall, it was demonstrated that disruption of purine biosynthesis by deletion of *purK* can trigger increased wrinkling of the macrocolony surface in a σ^B-independent manner.

II.1.4 Oxygen sensing modulates regulation of macrocolony biofilm formation

The gene for the response regulator *srrA* was the only candidate from the NTML screen with a described function in gene regulation. SrrA operates in coaction with its cognate histidine kinase SrrB to alter gene expression in response to low oxygen concentrations (Pragman et al., 2004, Ulrich et al., 2007). The genes *srrA* and *srrB* are encoded in a two-gene-operon (Throup et al., 2001, Yarwood et al., 2001)(Fig. 21a).

The macrocolonies of *ΔsrrA* displayed a flat surface without the characteristic wrinkles found in the WT (Fig. 21b). Despite the lack of wrinkles, the surface of the macrocolonies was rigid and fragmented upon dispersal with blunt objects (Fig. 21b). The WT-like macrocolony morphology was restored by expression of *srrA* from a neutral locus (Fig. 21c).

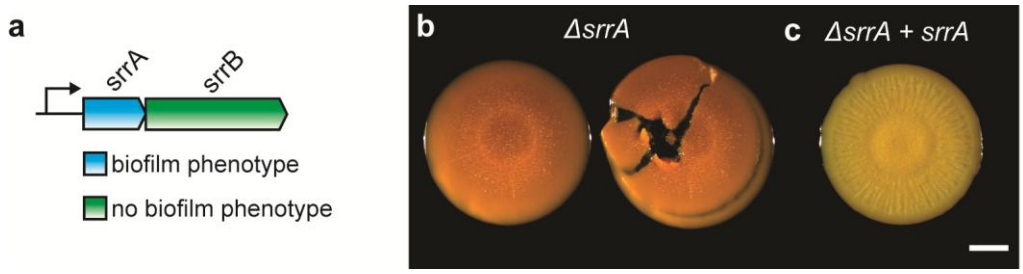

Fig. 21: Macrocolony biofilm defect in *ΔsrrA.*

(**a**) Genetic organization of the operon *srrAB*. (**b**) *ΔsrrA* forms unwrinkled macrocolonies that resist dispersal. (**c**) Expression of *srrA* restores wrinkling of the macrocolony. Macrocolonies in (**b**,**c**) were imaged after 5 days. Scale bar, 2 mm.

To investigate the stress response in absence of SrrA, the activity of the stress-induced alternative sigma factor σ^B was analyzed in macrocolonies of *ΔsrrA*. In addition to promoting biofilm formation in macrocolony aggregates, σ^B promotes the production of the pigment staphyloxanthin by activating the *crt* operon (Pelz et al., 2005). In return, the pigment can be quantified to obtain an approximation for σ^B-activation. Production of staphyloxanthin is increased in macrocolonies due to the magnesium-dependent σ^B-activation (Garcia-Betancur et al., 2017). The staphyloxanthin was extracted from the macrocolonies using methanol. The absorption of the pigment at 463 nm was normalized to the cell density of the resuspended macrocolony, which was determined by the optical density at 600 nm. The macrocolonies of *ΔsrrA* produced 64% and 73% more staphyloxanthin than the WT after 3 and 5 days, respectively (Fig. 22a). The increase of pigment production between the time points was 11% in *ΔsrrA* compared to only 6% in the WT. These results indicated a stronger activation of σ^B in SrrA-depleted macrocolonies.

The biofilm-promoting activity of σ^B in macrocolony aggregates depends on repression of the QS system Agr (Garcia-Betancur et al., 2017). As Agr controls the production of hemolytic toxins, the lytic effect of *S. aureus* on erythrocytes on blood agar plates or in solution can be quantified to measure Agr activity (Haque and Baldwin, 1964, Yarwood and Schlievert, 2003). The experimental conditions to quantify Agr activity should be chosen with care due to the regulation of the system by environmental stimuli (Burnside et al., 2010, Regassa et al., 1992). To mimic conditions in macrocolony aggregates, Agr activity was measured in

submerged biofilms in TSBMg medium. In this set-up, the deletion of *srrA* did not affect the production of hemolytic toxins. Similarly, the *ΔsrrA ΔsigB* double mutant had hemolytic activity comparable to the WT, whereas hemolysin production was increased more than 4-fold in a *ΔsigB* strain. Hence, the additional deletion of *srrA* neutralized the positive effect that the deletion of σ^B has on Agr activity (Fig. 22b).

The interplay of *srrA* with the σ^B-Agr-pathway in macrocolony aggregates was further studied by analyzing the macrocolony morphology of double mutants of *ΔsrrA* with either of the aforementioned regulators. *ΔsrrA ΔsigB* had a greater biofilm defect than the single mutants. The rigidity of the macrocolonies was reduced and only the core resisted biofilm dispersal (Fig. 22c). The biofilm defect of *ΔsrrA* was partially rescued by the additional deletion of *agr*. The *ΔsrrA Δagr* double mutant formed a macrocolony with intermediate levels of macrocolony surface wrinkling (Fig. 22c).

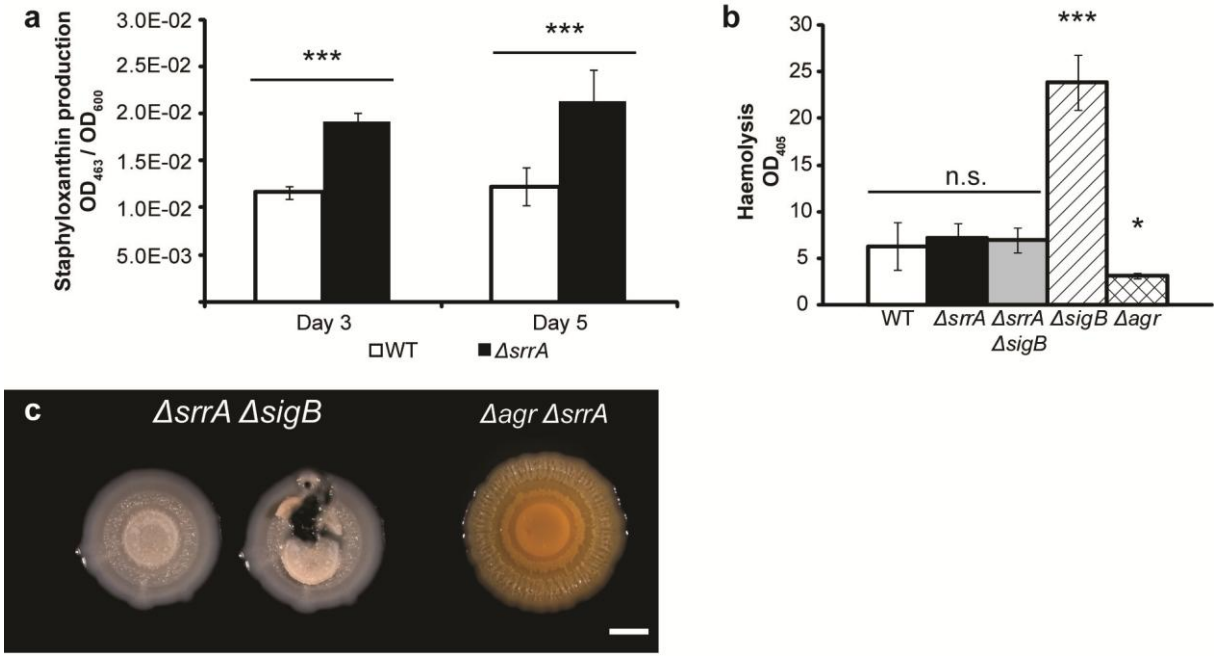

Fig. 22: Deletion of *srrA* modulates σ^B and Agr activity.

(a) Staphyloxanthin production is increased in *ΔsrrA* macrocolonies in mid- and late-stage biofilm development. (**b**) Hemolysis of submerged biofilms in TSBMg in *ΔsrrA* and *Δagr* differs from WT (**c**) Biofilm defect of *ΔsrrA ΔsigB* macrocolonies is stronger than in the single mutants (left). Only the core of the macrocolony resists dispersal (middle). Wrinkling of macrocolony is partially restored in *ΔsrrA Δagr* (right). Graphs in (**a**,**b**) show mean ± SD of 3 independent experiments. P values were calculated using unpaired Student's t-test using WT as reference. * p≤0.05, *** p≤0.001, n.s. not significant. Macrocolonies in (**c**) were imaged after 5 days. Scale bar, 2 mm.

In conclusion, the disruption of an oxygen-sensing TCS caused a biofilm defect in the macrocolony model, which could be reversed by Agr deletion. The deletion of *srrA* affected σ^B activity and Agr activity in a *ΔsigB* background. These results indicate that the TCS SrrAB could function as a modulator of the σ^B-Agr-signaling cascade in the macrocolony biofilm model.

II.1.5 Crucial role for gluconeogenesis in macrocolony matrix production

Twenty-one percent of the candidates identified in the screen pertained genes involved in carbohydrate metabolism, including gluconeogenesis. The first reaction step of this pathway is catalyzed by the enzyme PckA (Scovill et al., 1996). The corresponding gene *pckA* is transcribed monocistronically from the chromosome (Fig. 23a). The strain *ΔpckA* formed broadly-spreading macrocolonies that appeared thin and without a 3D-structure outside the core area (Fig. 23b).The macrocolonies resisted dispersal with blunt objects (Fig. 23b). The WT-like structure of the macrocolony was restored by expression of *pckA* from a neutral locus (Fig. 23c). As for *ΔpurK* and *ΔsrrA*, it was also determined if σ^B was involved in the *ΔpckA* phenotype. The *ΔpckA ΔsigB* strain formed a flat macrocolony without 3D-structure, which resembled the *ΔpckA* phenotype (Fig. 23d). Therefore, it was concluded that σ^B activation was not involved in the *ΔpckA* macrocolony phenotype.

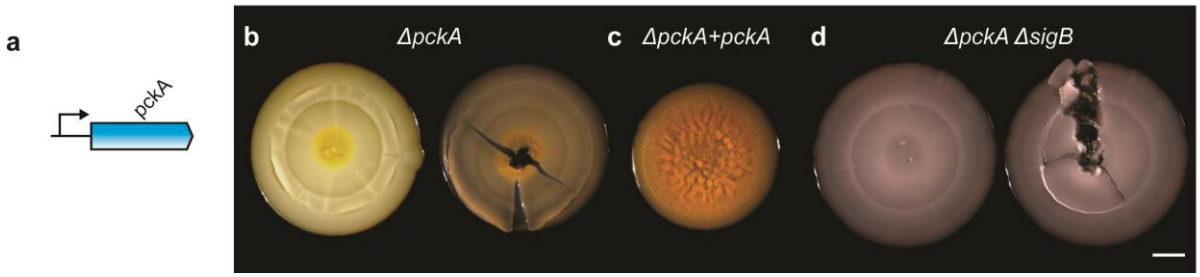

Fig. 23: Gluconeogenesis-deficiency compromises macrocolony structuring

(**a**) *pckA* is transcribed monocistronically. (**b**) *ΔpckA* forms thin macrocolony without structuring of surface (left). *ΔpckA* macrocolony resists mechanical dispersal (right). (**c**) Expression of *pckA* restores WT-like macrocolony morphology. (**d**) *ΔpckA ΔsigB* macrocolony morphology resembles *ΔpckA*-phenotype. Macrocolonies were imaged after 5 days. Scale bar, 2 mm.

In addition to the parchment-like phenotype, *ΔpckA* macrocolonies displayed another phenotype which indicated a defect in macrocolony aggregation. Upon exposure to mild

stress, i.e. the heat from a light source, *ΔpckA* macrocolonies spontaneously developed cracks in their structure. The fragmentation commenced within 15 s of heat exposure and expanded over time. Fragments that were detached from the agar and curled upwards could be observed (Fig. 24). WT macrocolonies did not show any observable reactions to heat exposure (Fig. 24).

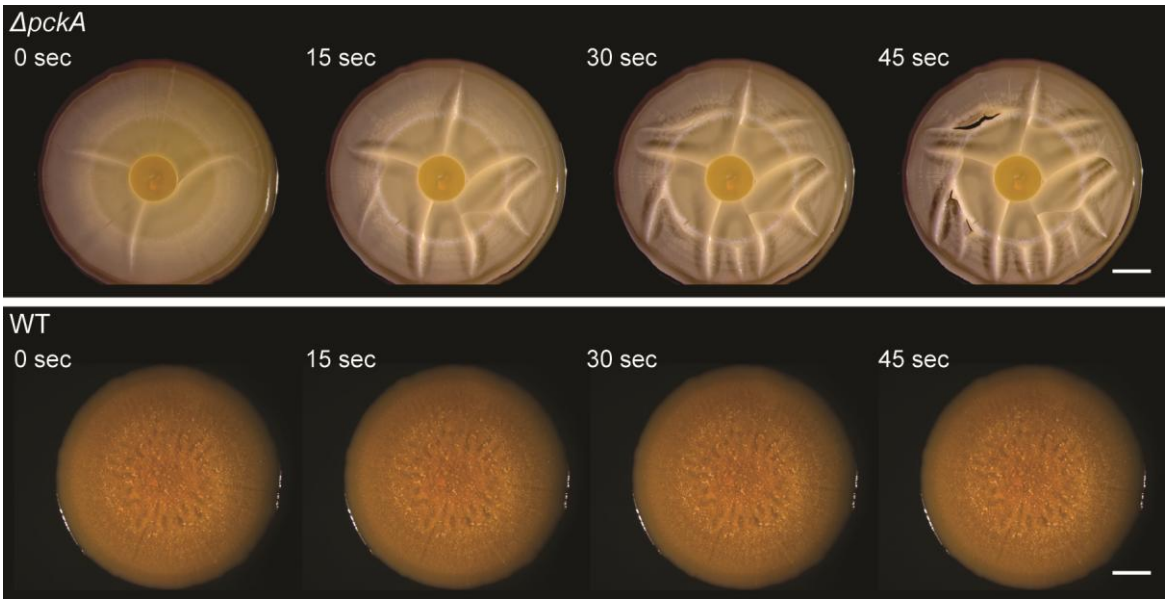

Fig. 24: Heat exposure damages *ΔpckA* macrocolony integrity.

ΔpckA macrocolony spontaneously fragments upon heat exposure and partially detaches from surface (upper panel). WT macrocolony is not affected by heat exposure (lower panel). Macrocolonies were imaged after 7 days. Scale bar, 2 mm.

The brittle phenotype of *ΔpckA* macrocolonies led to the question if the extracellular matrix was altered in the mutant in comparison to the WT. To analyze the ECM composition, it was extracted from mature macrocolonies of *ΔpckA* and WT. High NaCl concentrations disrupt the electrostatic interaction between the ECM and cells, hence enabling separation of the ECM and cellular material (Chiba et al., 2014). The principal ECM components – proteins, eDNA and saccharides – were quantified separately using spectrophotometric methods. Levels of proteins, eDNA and saccharides in 5-day-old macrocolonies were reduced by 67%, 71% and 66%, respectively, in the mutant compared to the WT (Fig. 25). The reduced deposition of extracellular material to build the matrix in *ΔpckA* macrocolonies could provide an explanation for the sensitivity of the biofilms against heat stress.

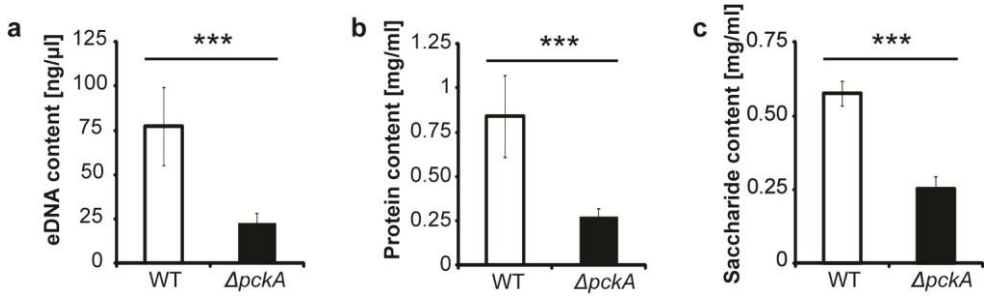

Fig. 25: Reduced matrix production in *ΔpckA* macrocolonies.

(**a**) eDNA content in matrix of *ΔpckA* macrocolonies is reduced by 71% compared to WT. (**b**) Protein content in matrix of *ΔpckA* macrocolonies is reduced by 67% compared to WT. (**c**) Saccharide content in matrix of *ΔpckA* macrocolonies is reduced by 66% compared to WT. Matrix was extracted from macrocolonies after 5 days. Graphs show mean ± SD of 5 independent experiments. P values were calculated using unpaired Student's t-test. *** p≤0.001

Growth of WT and *ΔpckA* was compared in various growth conditions to determine if the mutant had a generalized fitness defect or only a specific defect related to matrix production in the biofilm. The complex medium TSB, which is the basis of the growth medium of the macrocolony biofilm assay, contains 0.25% glucose as a carbon source. In contrast, lysogeny broth (LB) is a complex medium without glucose as a carbon source. LB was supplemented with different carbon sources to determine the growth of *ΔpckA* in conditions requiring gluconeogenesis. In TSB and LB supplemented with 0.5% glucose, growth of *ΔpckA* was comparable to WT or accelerated during exponential and stationary growth phases, respectively (Fig. 26a,c). In LB without additional glucose, the optical density of *ΔpckA* cultures lagged behind WT cultures (Fig. 26b). A similar effect was observed when sodium pyruvate was added to LB as a carbon source (Fig. 26d). To use sodium pyruvate for gluconeogenesis, the enzymatic activity of PckA is required; hence *ΔpckA* can not metabolize it. The dependency of *ΔpckA* on glucose supplementation was also noted in a competition experiment between the mutant and WT. Cultures were initiated with equal ratios of both strains. The abundance was traced over 48 h by taking samples at the indicated time points and plating serial dilutions. TSB plates were used to determine the total colony forming units (CFU) of both strains and selections plates only allowing growth of *ΔpckA* were used to determine the share of the mutant. For 6 h, the ratios remained stable, but after 24 h

the relative abundance of the WT increased over the mutant (Fig. 26e). The displacement of the mutant from the culture coincided temporally with the depletion of glucose from the growth medium (Ledala et al., 2014). In conclusion, *ΔpckA* has a fitness defect in long-term growth, which could contribute to the extracellular matrix deficiency and macrocolony phenotype.

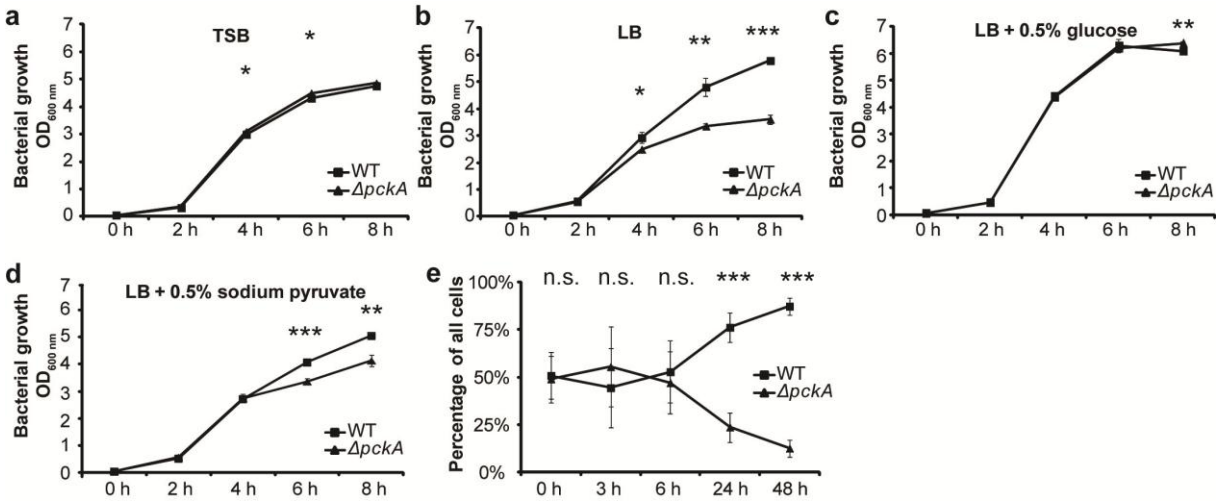

Fig. 26: Glucose positively affects growth of *ΔpckA.*

(**a**) WT and *ΔpckA* grow similarly in TSB medium. (**b**) *ΔpckA* grows less than WT in LB medium. (**c**) Glucose supplementation to LB medium improves *ΔpckA* growth to WT levels. (**d**) Supplementation of sodium pyruvate to LB medium is not sufficient to compensate the growth defect of *ΔpckA*. (**e**) *ΔpckA* is outcompeted by WT after 24h in TSB medium. Graphs in (**a**, **b**, **c**, **d**) show mean ± SD of 3 independent experiments. Graphs in (**e**) show mean ± SD of 10 cultures. P values were calculated using unpaired Student's t-test. * p≤0.05, ** p≤0.01, *** p≤0.001, n.s. not significant.

II.1.6 Virulence of biofilm regulators in a non-vertebrate *in vivo* model

Staphylococcal mutants of the newly identified biofilm regulators PckA, PurK and SrrA were injected into *Galleria mellonella* larvae to compare their pathogenic potential to the WT strain. *G. mellonella* larvae are frequently used as a non-vertebrate infection model to study pathogenic bacteria and antibacterial compounds because they can be held at 37°C, thus mimicking the human body temperature (Desbois and Coote, 2011, Koch et al., 2014a, Ramarao et al., 2011). Similar to the human innate immune system, the insect larvae use antimicrobial peptides and phagocytosing cells against pathogenic bacteria (Hoffmann, 1995, Mullett et al., 1993).

G. mellonella in the last larval stage (~250 mg body weight) were manually injected with 1.5 x 10^6 CFU in the last proleg, while negative controls were injected with saline solution. Survival rates of the larvae were determined after 24 h and 48 h. Infection with WT resulted in larvae survival rates of 40% and 15% after 24 h and 48 h, respectively. Similarly, infection with mutant strains *ΔpckA* and *ΔpurK* resulted in larvae survival rates of 30% and 10% at these time points. The differences between the mutants and the WT were not statistically significant ($p > 0.05$). The cohorts of larvae infected with *ΔsrrA* had higher survival rates as 80% survived after 24 h and 40% after 48 h. The attenuation of virulence was calculated to be statistically significant after 24 h, but not after 48 h (Fig. 27). In this non-vertebrate infection model, virulence did not correlate with increased and decreased biofilm formation in the macrocolony model.

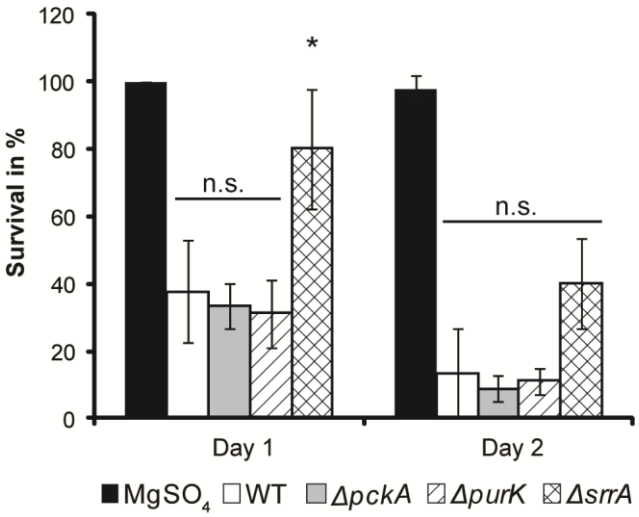

Fig. 27: Absence of SrrA attenuates virulence of *S. aureus*.

Injection of *Galleria mellonella* larvae with 1.5 x 10^6 CFU of WT, *ΔpckA*, *ΔpurK* and *ΔsrrA*. Strain *ΔsrrA* is attenuated in virulence after 24 h but not after 48 h. *ΔpckA* and *ΔpurK* virulence is similar to the WT strain. Graphs show percent survival as mean ± SD of 3 independent experiments. P values were calculated using unpaired Student's t-test with WT as the reference. * p≤0.05, n.s. not significant.

II.2 Inter-strain competition in *S. aureus* macrocolonies

II.2.1 Interstrain interactions in the macrocolony biofilm model

Previous research using the macrocolony model of biofilm formation in *S. aureus* demonstrated how bacterial competition can lead to the emergence of new genetic variants and phenotypic traits (Koch et al., 2014b). The colonization with *S. aureus* in the human nares is predominantly monoclonal, contrasting the polyclonal colonization by other pathogens (Cespedes et al., 2005, St Sauver et al., 2000). This suggests the presence of mechanisms that prevent co-colonization with several strains. Therefore, this section of the thesis analyzes the engagement of different clinical isolates of *S. aureus* in interstrain competition in the macrocolony biofilm model. The experimental approach was to spot macrocolony aggregates with a mixed community of the commonly used lab strains Newman and USA300 (Duthie and Lorenz, 1952, Gonzalez et al., 2005). The resulting macrocolonies were analyzed for changes on the macroscopic level or newly emerging strains.

The strains Newman and USA300 are representatives of methicillin-susceptible (MSSA) and methicillin-resistant (MRSA) *S. aureus* isolates, respectively. Based on multilocus sequence typing (MLST) of conserved housekeeping genes, the strains are closely related as they both have sequence type 8 (ST8) (Baba et al., 2008, Diep et al., 2006, Enright et al., 2000, Highlander et al., 2007). Despite this similarity, the strains differ in genetic and phenotypic characteristics such as antibiotic susceptibility (Gonzalez et al., 2005), prophages (Bae et al., 2006, Diep et al., 2006), signaling networks (Steinhuber et al., 2003) as well as capsule and toxin production (Boyle-Vavra et al., 2015, Brown et al., 2012).

As shown in the previous chapter, Newman and USA300 both form symmetrically structured and pigmented macrocolonies on TSBMg (Fig. 28a). In a mixed community of the strains on TSBMg, the core was pigmented homogenously but the outer parts of the macrocolony showed differentially pigmented rays (Fig. 28a, arrows). Interestingly, within the stronger pigmented rays, irregular lines of darker discolorations were observed. The macrocolonies appear lytic in these areas and the structuring of the surface is also decreased compared to

the unaffected areas of the macrocolony. As the nature of social interactions in bacteria is sensitive to fluctuations of the environmental conditions, it was assessed if the biofilm-like lifestyle in the macrocolonies influenced the interstrain interactions (Liu et al., 2017a). To this end, Newman and USA300 were grown in monoculture as well as mixed communities on TSB agar. The monoculture colonies were homogenously pigmented and their surface did not appear structured. Similarly, the mixed community of the strains was homogenously pigmented on its entire surface and lacked lytic areas (Fig. 28b).

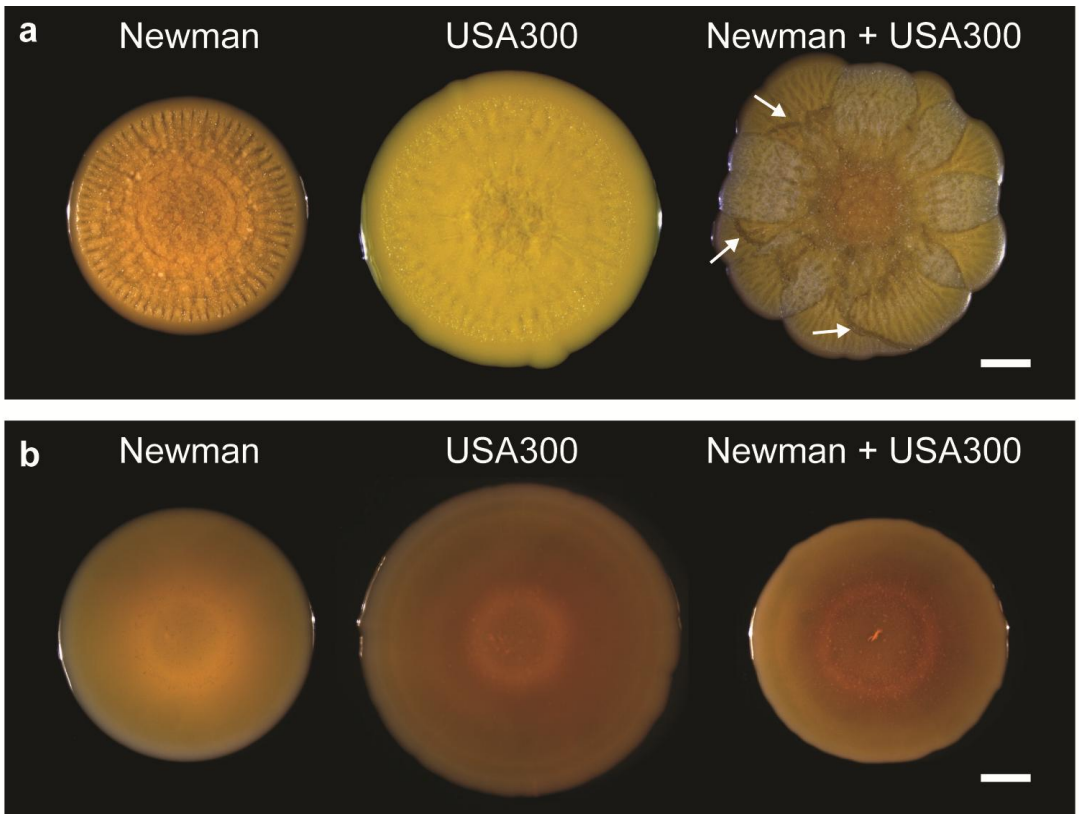

Fig. 28: Mixed communities of Newman and USA300 develop lytic discolorations.
(**a**) Macrocolonies of Newman and USA300 are homogenously structured and pigmented. A mixed community of the strains develops differentially pigmented sectors and lytic discolorations that are characterized by a loss of the macrocolony structure (arrows). (**b**) Newman and USA300 form homogenously pigmented colonies without discernible structure on TSB. A mixed community does not display segments or lysis on TSB. Macrocolonies were imaged after 5 days. Scale bar, 2 mm.

In order to determine if one or both strains were affected by this phenomenon, mutants of the regulator σ^B were used to track strain lineage. These strains characteristically lack pigmentation (Nicholas et al., 1999). Mixed communities combining Newman *ΔsigB* with USA300 WT as well as Newman WT with USA300 *ΔsigB* were set to be able to trace the

lytic discolorations. In these mixed communities, the strains with a Newman background were not affected by lysis. In contrast, USA300 WT as well as USA300 *ΔsigB* appeared lytic in mixed communities with the Newman counterpart (Fig. 29). The lytic discolorations did not affect all sectors of USA300 in the mixed communities, but they were observed consistently. At the intersection between rays of Newman- and USA300-descent the lysis was terminated abruptly. No spreading of lysis into sectors of Newman was observed. On TSB, the strain lineage could be traced in the mixed communities but lytic areas were not observed (Fig. 29).

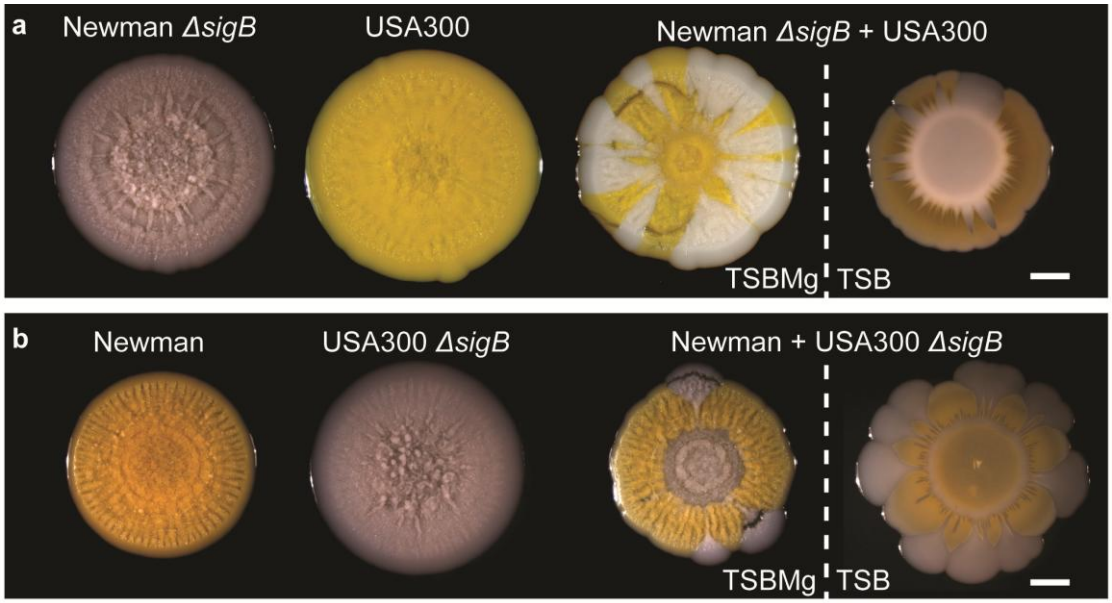

Fig. 29: Lytic discolorations can be traced to USA300 strain.

(a) Monoculture macrocolonies of Newman *ΔsigB* and USA300 on TSBMg are homogenously structured and colored. A mixed community of the strains develops lytic discolorations in the pigmented USA300 sectors but not in the unpigmented Newman *ΔsigB* sectors. Lysis is not observed on TSB. **(b)** Monoculture macrocolonies of Newman and USA300 *ΔsigB* on TSBMg are homogenously structured and colored. A mixed community of the strains develops lytic discolorations in the unpigmented USA300 *ΔsigB* sectors but not in the pigmented Newman sectors. Lysis is not observed on TSB. Macrocolonies were imaged after 5 days. Scale bars, 2 mm.

Notably, on TSB agar USA300 *ΔsigB* spread faster and displaced Newman WT at the outer sections of the mixed community. To a lesser extent, the formation of fast-spreading sectors by USA300 *ΔsigB* was also observed on TSBMg (Fig. 29b). The deletion of σ^B was previously linked to increased spreading and a competitive advantage (Koch et al., 2014b). As the lytic behavior of USA300 only occurred on biofilm-inducing TSBMg agar but not on

TSB agar, the process was deemed to be biofilm-specific and the following experiments were performed on TSBMg agar, unless indicated otherwise.

In order to rule out that the deletion of σ^B alone had a detrimental effect on colony integrity, mixed communities with WT and *ΔsigB* in the same strain background were made. These mixed communities showed pattern of radiation between the different variants, but no lytic discolorations could be observed (Fig. 30). Thus, the occurrence of lytic sectors in the macrocolonies was hypothesized to be a consequence of lysis in the USA300 community by the competing strain Newman independent of the action of σ^B.

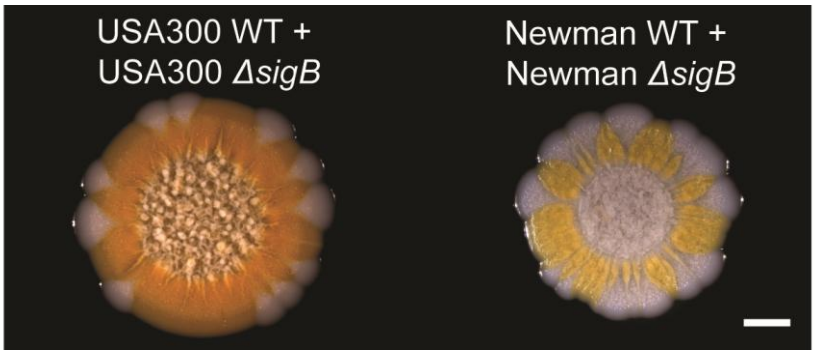

Fig. 30: Lysis of macrocolonies is independent of the deletion of *sigB*.
Mixed communities of USA300 WT and USA300 *ΔsigB* (left) as well as Newman WT and Newman *ΔsigB* (right) contain pigmented and unpigmented sectors, but no lysis is observed in either combination. Macrocolonies were imaged after 5 days. Scale bar, 2 mm.

For a better understanding of the underlying processes that cause lysis of the USA300 sectors, a macrocolony that contained a mixed community of Newman *ΔsigB* and USA300 was tracked for 5 days. In this experiment, small discolorations were visible after 24 h (Fig. 31). The formation of defined sectors of the strains could be observed after 3 days. Thus, the processes that underlie the lysis of USA300 already occur in early stages of macrocolony development. Additionally, the experiment confirmed that lysis was contained to USA300 and never affected Newman at all stages of macrocolony development.

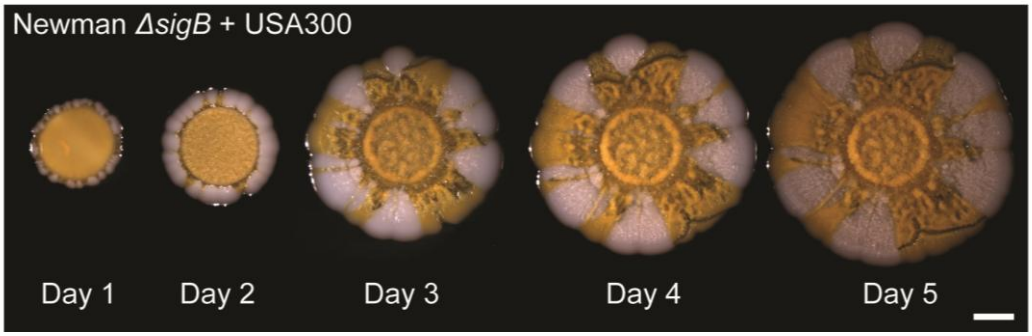

Fig. 31: Lytic discolorations appear early in development of mixed communities.
Discoloration in mixed community of Newman *ΔsigB* and USA300 can be observed after 1 day already. Defined pigmented and pigment-deficient sectors that can be attributed to USA300 and Newman *ΔsigB*, respectively, become apparent after 3 days of development. Lytic discolorations are limited to USA300 sectors. Macrocolonies were imaged for 5 consecutive days. Scale bar, 2 mm.

II.2.2 Identification of phages as lysis-inducing particles

Contact-dependent as well as contact-independent mechanisms have been described in the killing of *S. aureus* cells by bacterial competitors (Cao et al., 2016, Khan et al., 2016). To determine if contact-dependency was present in the observed lysis of USA300, monoculture macrocolonies were exposed to cell-free culture supernatant of the competitor. As seen in Fig. 31, the lysis-triggering of USA300 occurred early in macrocolony development and caused visible lysis after 24 h already. Consequently, the macrocolonies were exposed to the culture supernatant after 8 h. The supernatants were collected from cultures that were grown in TSB medium for the same duration and sterile filtered to remove all cells. Macrocolonies of USA300 that were spotted with supernatant of Newman WT and *ΔsigB* cultures showed lytic discolorations despite not having been exposed to cells of the Newman lineage (Fig. 32). Spotting the supernatant of USA300 cultures or fresh TSB medium on the macrocolonies did not have this effect. Hence, the lysis-causing process is not dependent on the presence of Newman cells but can also be triggered by the supernatant of Newman cultures. In contrast, Newman macrocolonies were not affected by the supernatant of Newman or USA300 cultures. Similarly to the results of mixed communities on TSB agar, neither USA300 nor Newman colonies were susceptible to supernatant-triggered lysis when grown on TSB agar without magnesium supplementation (Fig. 32).

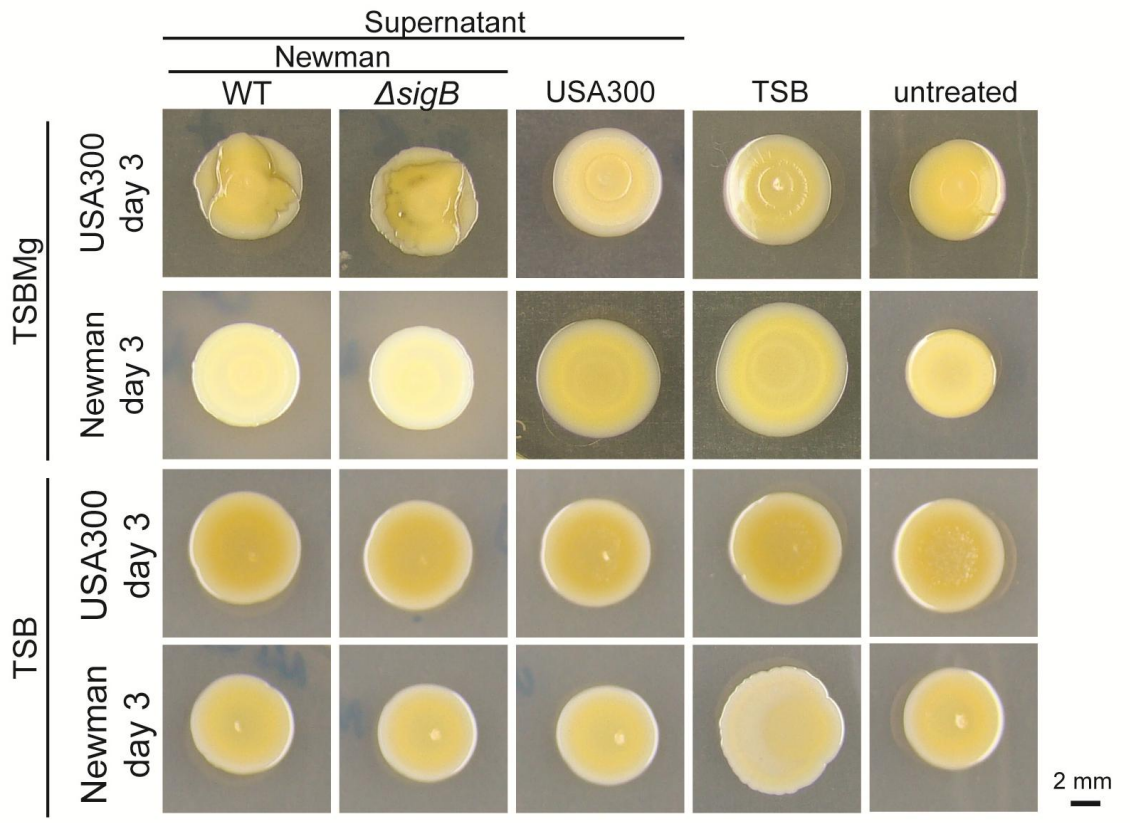

Fig. 32: Supernatant of Newman cultures is active against USA300 macrocolonies on TSBMg.

USA300 macrocolonies on TSBMg agar treated with Newman supernatants show signs of lysis (first row). USA300 supernatant and fresh medium have no effect on macrocolonies. Newman macrocolonies on TSBMg agar are not affected by any treatment in this assay (second row). Colonies of both strains on TSB agar do not form lytic sectors after exposure to culture supernatants (third and fourth row). Macrocolonies were imaged after 3 days. Scale bar, 2 mm.

Modifications of the supernatant assay were used to test the effect of different treatments of the supernatant on the induction of lysis in order to characterize the lytic agent. The lytic agent in Newman supernatant remained active after incubation at 37°C for 20 min as well as after proteinase K treatment at this temperature for 20 min (Fig. 33). In contrast, Newman supernatant that was incubated at 58°C for 20 min did not have a lytic effect on USA300 macrocolonies anymore. Hence, the trigger of the lytic processes in USA300 macrocolonies resists proteolysis by proteinase K but is inactivated at 58°C.

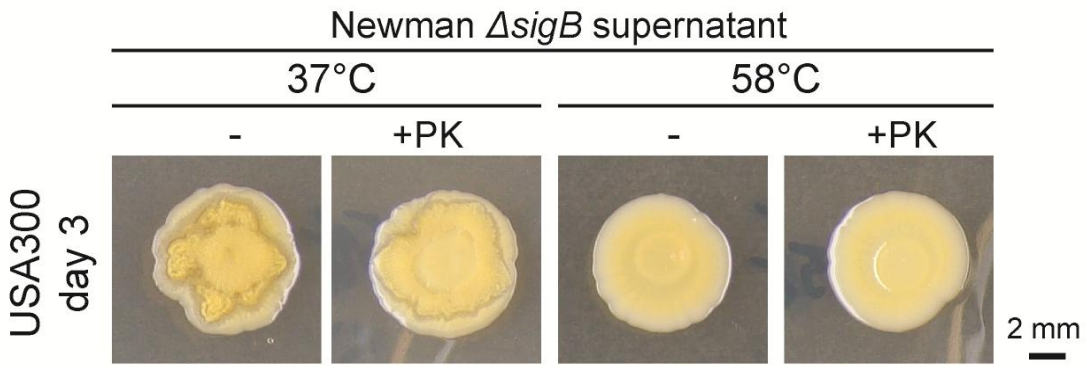

Fig. 33: Lytic agent withstands proteolysis and is heat-inactivated.

The lysis-triggering agent in Newman *ΔsigB* supernatant withstands incubation at 37°C and proteinase K treatment. The agent can be heat-inactivated at 58°C. Supernatant was heated and treated with proteinase K (PK) for 20 min. Macrocolonies were imaged after 3 days. Scale bar, 2 mm.

To approach a further characterization of the lytic agent, the supernatant of Newman *ΔsigB* cultures in TSB was subjected to rate-zonal centrifugation on a sucrose gradient to estimate the size of the lytic agent (Brakke, 1953, Hirst and Cox, 1976). The components of the supernatant were loaded on a continuous 5-40% gradient and separated by ultracentrifugation overnight. The sucrose gradient was split into 10 fractions that were all tested for their effect on USA300 macrocolonies. The lowest fraction (10+), which also contained the pellet from the bottom of the tube, induced weak lytic sectors in the macrocolony (Fig. 34). Based on this finding, it was hypothesized that bacteriophages could be the lysis-causing agent in the supernatant. The genome of *S. aureus* Newman contains four prophages of the *Siphoviridae* family (Bae et al., 2006). The capsid head of the phage has a diameter of approximately 50 nm and contains 40-50 kb DNA (Bae et al., 2006). Therefore, phages are large particles, which likely migrate to low fractions or the pellet of the sucrose gradient.

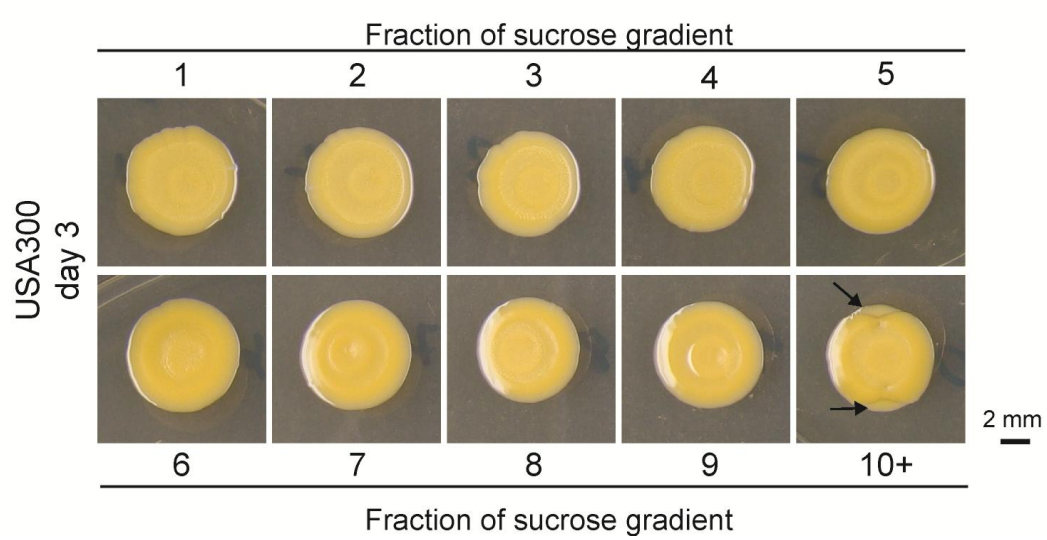

Fig. 34: Large particles identified as lysis-triggering agent.

Contents of Newman *ΔsigB* supernatant were separated by size on a sucrose gradient. Ten fractions were recovered and spotted onto USA300 macrocolonies. Fractions 1-9 were inactive against the macrocolonies. The lowest recovered fraction (10+) caused small lytic lesions in USA300 macrocolonies (arrows). Macrocolonies were imaged after 3 days. Scale bar, 2 mm.

The proteins from the fraction 10+ were precipitated and identified by mass spectrometry (MS) to determine if an accumulation of phage-related proteins could be found. The analysis yielded a list of 598 peptides that matched database entries for *S. aureus*. The best matches as well as phage-related matches identified by MS are presented in Table 2. Remarkably, only 4 phage-related proteins could be identified. In contrast, the comparison with a list of the most abundant proteins of *S. aureus* revealed great similarities between the datasets (Zuhlke et al., 2016). Zuhlke et al. have compiled a list of the most abundant proteins in three different growth phases. Eleven of the first 20 proteins identified by MS were also among the 50 most abundant proteins during at least one growth phase as described in this publication (Table 2) (Zuhlke et al., 2016). It is conceivable that the majority of MS-identified peptides result from debris of lysed cells as cell lysis occurs during the stationary growth phase of the culture (Patton et al., 2005).

#	Protein	Description
1	**FusA**	Elongation factor G
2	**GapA1**	Glyceraldehyde-3-phosphate dehydrogenase 1
3	**Tuf**	Elongation factor Tu
4	RpoC	DNA-directed RNA polymerase subunit beta
5	**Tkt**	Transketolase
6	**MetE**	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
7	RpoB	DNA-directed RNA polymerase subunit beta
8	**Pyk**	Pyruvate kinase
9	ClpC	ATP-dependent Clp protease ATP-binding subunit
10	ClpB	Chaperone protein ClpB
11	**PdhC**	Dihydrolipoamide acetyltransferase
12	PycA	Pyruvate carboxylase
13	**DnaK**	Chaperone protein DnaK
14	**GroL**	60 kDa chaperonin
15	**AcnA**	Aconitase
16	AlaS	Alanine—tRNA ligase
17	GyrA	DNA gyrase subunit A
18	ThrS	Threonine—tRNA ligase
19	SucC	Succinate--CoA ligase [ADP-forming] subunit beta
20	**PdhD**	Dihydrolipoyl dehydrogenase
⋮		
193	NWMN_1019	Phage head protein
339	NWMN_0294	Phage major head protein
481	SaurJH9_1070	Phage tail tape measure protein
494	NWMN_1812	Phage repressor

Table 2: Peptides identified in active fraction of Newman *ΔsigB* supernatant.

List of the first 20 peptides identified by MS in fraction 10+ of Newman *ΔsigB* supernatant as well as phage-related proteins that were identified in the analysis. Proteins highlighted in bold were also among the 50 most abundant proteins in at least one growth phase identified by Zuhlke et al., 2016.

Phages that infect and lyse bacteria can leave transparent plaques in a lawn of cells on a semisolid medium like agar (Sambrook et al., 1989). The plaque forming units (pfu) are frequently used to determine the phage titer in solutions. The release of bacteriophages in monoculture and mixed-community macrocolonies was assessed by the pfu recovered from

the aggregates. The relative phage release was calculated by determining the ratio of pfu to the CFU that estimate the amount of bacteria in the macrocolony. RN4220 was chosen as the susceptible acceptor strain in which the plaque formation was monitored. Phage release per cell in USA300 macrocolonies was found to be around 2×10^{-10} phages/cell on day 1 and continuously decline to 5×10^{-12} phages/cell on day 5 (Fig. 35). Phage release from Newman *ΔsigB* macrocolonies was higher with 10^{-4} phages/cell on day 1. The lowest phage release ratio in this strain was found on day 4 with 1.2×10^{-6} phages/cell. Mixed communities of Newman *ΔsigB* and USA300 had the highest phage release in the assay. The relative phage release from these macrocolonies was 3.7×10^{-2} phages/cell on day 1 and dropped to 2×10^{-4} phages/cell on day 4. The dynamics of Newman *ΔsigB* monocultures and mixed communities were similar, with the highest values on day 1, followed by a continuous decline to day 4 and a slight recovery on day 5 (Fig. 35). The quantification of phage release from macrocolonies showed an increase of the phages released per cell in mixed communities compared to monocultures. However, using this assay it is not possible to identify the mechanisms that were involved in the process. Furthermore, it is not clear whether the surplus of phages was released from one of the strains or from both. The fact that the phage titer of USA300 macrocolonies lies 5-6 log-fold lower than the titer of Newman *ΔsigB* macrocolonies raises the opportunity that RN4220 is more sensitive to phages released from Newman strains than from USA300.

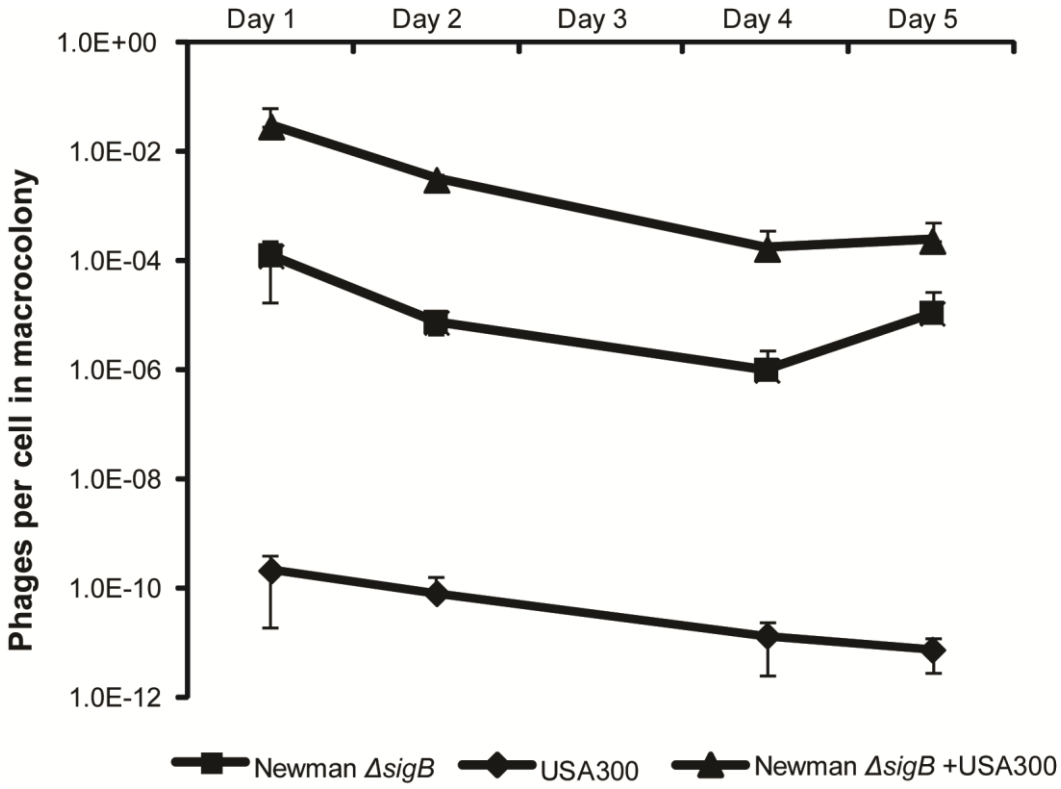

Fig. 35: Increased phage release in mixed communities.

Ratios of phage release and CFU were tracked as macrocolonies developed for 5 days. Mixed-community macrocolonies had higher ratio than Newman *ΔsigB* and USA300 monoculture macrocolonies. All tested communities had highest phage release at day 1 and subsequent decrease of phage release. Graphs show mean ± SD of 2 independent experiments.

It is characteristic for phage infections of bacteria that the process is self-maintaining but also self-limiting. Each lytic bacterial cell releases around 100 phage particles but usually phages do not drive bacterial populations to extinction (Heilmann et al., 2010, Kutter and Sulakvelidze, 2005). Based on the assumption that phages are involved in the lysis of USA300 macrocolonies, it was hypothesized that the lysis should persist in the absence of the trigger, i.e. the supernatant of Newman cultures. This hypothesis was tested with USA300 macrocolonies in which lysis was induced with supernatant of Newman *ΔsigB* (1st generation). After 3 days, material from lytic sectors and non-lytic control sectors was transferred to fresh TSBMg plates to start new macrocolonies (2nd generation). As expected, the macrocolonies that were started with material from control sectors formed macrocolonies without indications of lytic processes (Fig. 36). The irregular shape of the macrocolonies results from the non-uniform transfer of material. In contrast, macrocolonies that were started

with cellular material from lytic sectors, showed signs of lysis and loss of surface structure in the core of the macrocolony. The pattern of intact macrocolonies and lytic sectors was maintained in a 3rd generation of macrocolonies that were started with material of the 2nd generation. In conclusion, the experiment showed that the lysis of the USA300 macrocolonies involves a process that is self-maintaining and repeatedly causes lysis in macrocolonies that contain material of lytic macrocolonies.

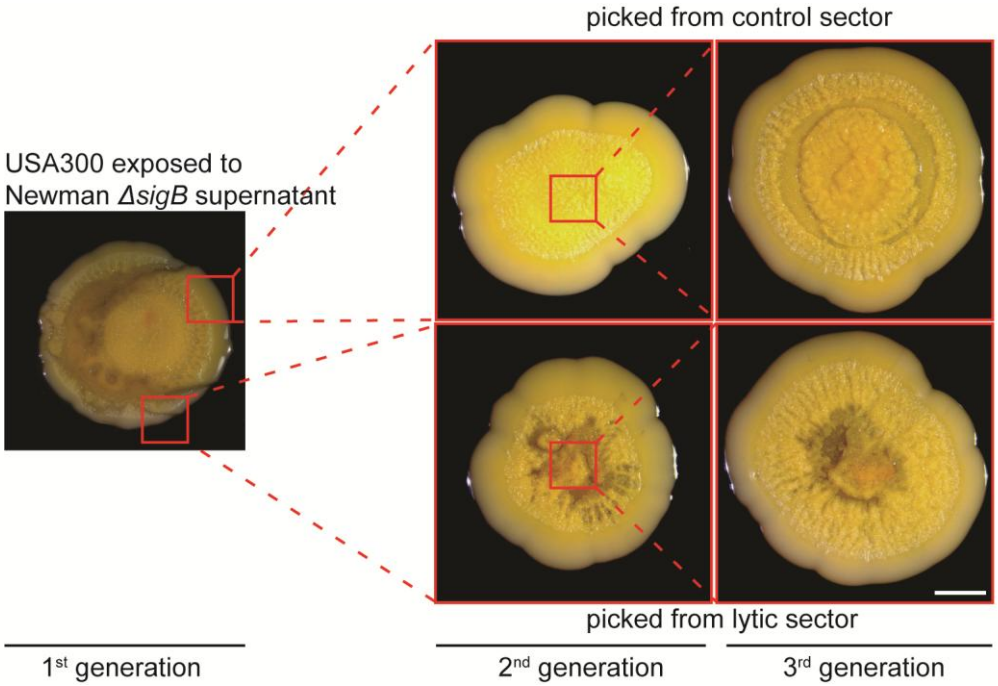

Fig. 36: Lytic phenotype is reproducible over 3 generations.

Lytic and intact macrocolony characteristics in USA300 are preserved when the respective sectors of the macrocolonies are transferred to form new macrocolonies. 1st generation denotes macrocolonies that were started from dense suspensions of cells and treated with Newman *ΔsigB* culture supernatant. 2nd generation macrocolonies were started by picking material from 1st generation macrocolonies after 3 days and transferring it to new plates. 3rd generation macrocolonies were started by picking material from 2nd generation macrocolonies after 3 days and transferring it to new plates. 1st generation macrocolonies were imaged after 3 days. 2nd and 3rd generation macrocolonies were imaged after 4 days. Images are representative for the respective generations. Scale bar, 2 mm.

The experimental approaches to identify the lysis causing trigger in the supernatant of Newman cultures gave several indications that phages were involved in the process. To confirm the bacteriophagic nature of the trigger, a system with phage-cured strains that were artificially re-transduced with phages was used. The strain RN450 (NCTC8325-4) has been cured of the prophages that are found in the genome of its parent strain RN1 (NCTC8325)

(Novick, 1967, Novick and Richmond, 1965). Variants of this strain that were transduced with φ11, φ80α or φ85 have been generated previously (Maiques et al., 2006, Selva et al., 2009). The supernatant of the parent strain and the variants was tested for the ability to cause lysis in macrocolonies of USA300 and Newman similarly to the previous supernatant experiments. As expected, the supernatant of the parent strain RN450, which does not carry prophages in the genome, was inactive against macrocolonies of Newman and USA300 (Fig. 37). In contrast, the supernatants of RN450 φ11 and RN450 φ85 lysed USA300 macrocolonies similarly to Newman supernatant. The variant RN450 φ80α was inactive against these macrocolonies. Newman macrocolonies were lysed by supernatants of RN450 φ80α and RN450 φ85, but not by RN450 φ11. These results show that lysis of macrocolonies is not determined by the lysis causing strain *per se* but rather by the prophages harbored in the strains. Furthermore, the appearance of lysis in macrocolonies depends on the susceptibility of the strain to the specific phage. In the competition experiments between Newman and USA300, USA300 is susceptible to one or several of the phages that are released by its competitor, causing the lytic appearance of the macrocolonies.

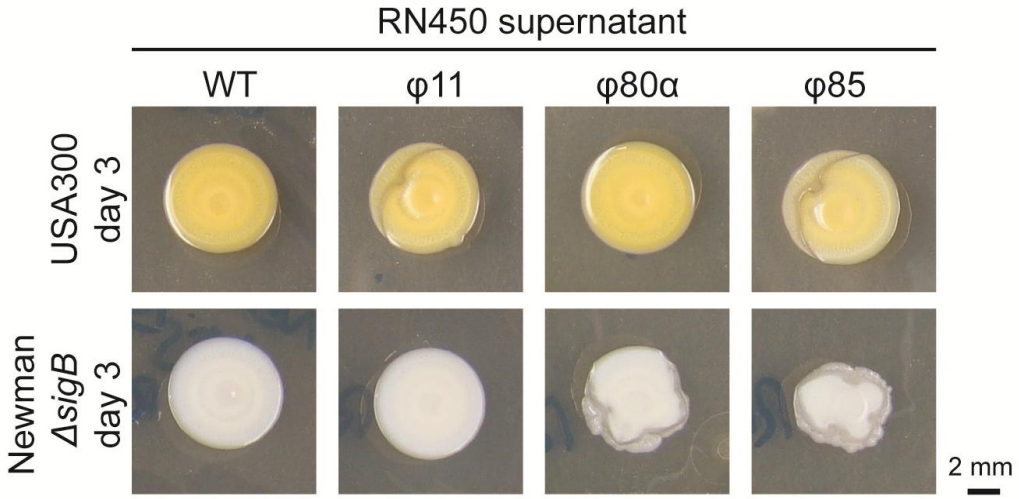

Fig. 37: Phage type rather than its origin determines lysis.

Variants of RN450 with different prophages diverge in their ability to trigger lysis in macrocolonies. USA300 macrocolonies (upper panel) are lysed by RN450 φ11 and RN450 φ85. Newman *ΔsigB* macrocolonies are lysed by RN450 φ80α and RN450 φ85. Macrocolonies were imaged after 3 days. Scale bar, 2 mm.

II.2.3 Exploration of lysis induction and resistance mechanisms

In a similar competition experiment, it was found that *Streptococcus pneumoniae* can induce lysis in *S. aureus* in a "remote-controlled" manner (Selva et al., 2009). In this study, H_2O_2 was identified as the trigger molecule to activate staphylococcal lysis dependent on RecA-mediated SOS response and prophage induction (Selva et al., 2009). To study if a similar mode of action was in play in our interstrain staphylococcal competition experiments, the role of H_2O_2 and RecA was assessed. Cultures of USA300 were grown to mid-exponential phase, H_2O_2 was added and incubation continued for 2 h. Control cultures were supplemented with H_2O. The final concentration of 1% H_2O_2 was determined as the maximum concentration from which CFU could be recovered. The exposure to 0.1% and 1% H_2O_2 did not induce the formation of lytic sections in USA300 macrocolonies (Fig. 38a). Similarly, spotting the same concentrations of H_2O_2 onto preformed macrocolonies in the manner of the supernatant assay did not induce lysis in USA300 or Newman (Fig. 38b).

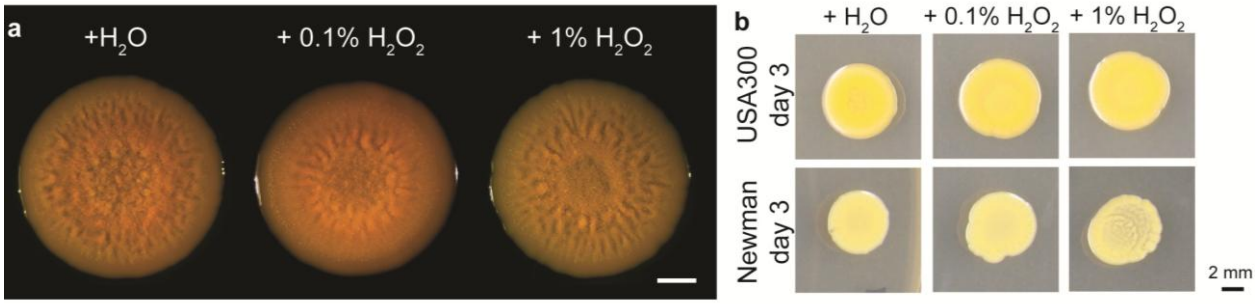

Fig. 38: Macrocolony lysis is not induced by H_2O_2-treatment.

(a) USA300 precultures treated with different concentrations of H_2O_2 (middle and right) do not give rise to macrocolonies with an altered morphology compared to control-treatment with H_2O (left). **(b)** Spotting H_2O_2 onto macrocolonies does not induce lysis in USA300 or Newman macrocolonies. Macrocolonies in **(a)** and **(b)** were imaged after 5 and 3 days, respectively. Scale bars, 2 mm.

To assess whether the macrocolony lysis was dependent on the SOS response, the effect of interstrain competition on a *recA* mutant, which is defective in the SOS response, was tested (Bisognano et al., 2004). The *recA* transposon mutant from the NTML was grown in a mixed community with Newman *ΔsigB*. For reference, the parent strain USA300-JE2 was also grown in competition with Newman *ΔsigB*. The mixed community of USA300-JE2 WT with Newman *ΔsigB* showed the same lytic behavior of the USA300 lineage that was observed

with the isolate used in the previous experiments (Fig. 29a and Fig. 39). The deletion of *recA* did not affect outcome of the competition as the same lytic behavior of the USA300 lineage was found. In conclusion, the lysis-causing mechanisms in the interspecies competition with *S. pneumoniae* and intraspecies competition between *S. aureus* isolates are distinct, as the role for H_2O_2 and RecA could not be confirmed in the intraspecies model of this study. These results indicate that the biofilm-specific lysis of USA300 promoted by Newman phages is independent of the SOS response.

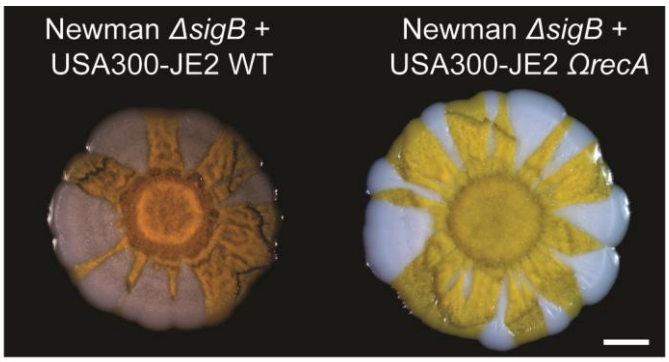

Fig. 39: RecA-deficient USA300-JE2 is susceptible to lysis.

USA300-JE2 macrocolonies lyse in a similar manner to USA300_TCH1516 when grown in a mixed community with Newman *ΔsigB* (left). Truncation of *recA* by a transposon insertion does not change the outcome of the competitive interaction (right). Macrocolonies were imaged after 5 days. Scale bar, 2 mm.

For the understanding of the mode of action of antibacterial processes, it can be helpful to study possible resistance mechanisms. Recently, a eukaryotic-like serine-threonine kinase, Stk2, was described to protect *S. aureus* communities from phage infections by triggering cell death of individual infected cells before phages can replicate in them (Depardieu et al., 2016). This defense strategy against phage infections is called abortive infection (Chopin et al., 2005). The gene *stk2* is found in the MRSA strain N315, but neither Newman nor USA300 carry the gene (Didier et al., 2010, Kuroda et al., 2001). To confirm that phage-infections are responsible for the lytic macrocolony phenotype in mixed communities, it was assessed if heterologous expression of *stk2* could prevent the phenotype. As Stk2 efficiently protects from phage infections, it was not possible to generate a phage lysate of the *stk2*-expressing RN4220 strain and the construct could not be shuttled to Newman or USA300. Therefore, the protective effect was assessed comparing RN4220 WT and RN4220 P_{stk2}-

stk2. Macrocolonies of RN4220 WT showed distinct lytic sectors upon exposure to supernatant of Newman WT and Newman *ΔsigB* cultures. Macrocolonies of the RN4220 strain that produced Stk2 were not affected by the supernatants (Fig. 40). Additionally, untreated macrocolonies of RN4220 P_{stk2}-*stk2* showed changes in the morphology compared to the WT. The core of the macrocolony showed distinct bulges that were absent in the WT, implying that the serine-threonine kinase has further effects in the macrocolonies in addition to abortive infection (Fig. 40). These results indicate that abortive infection is a protection mechanism against biofilm-specific lysis and thus contribute to the evidence that phages are involved in causing lysis in competing *S. aureus* strains in a biofilm setting.

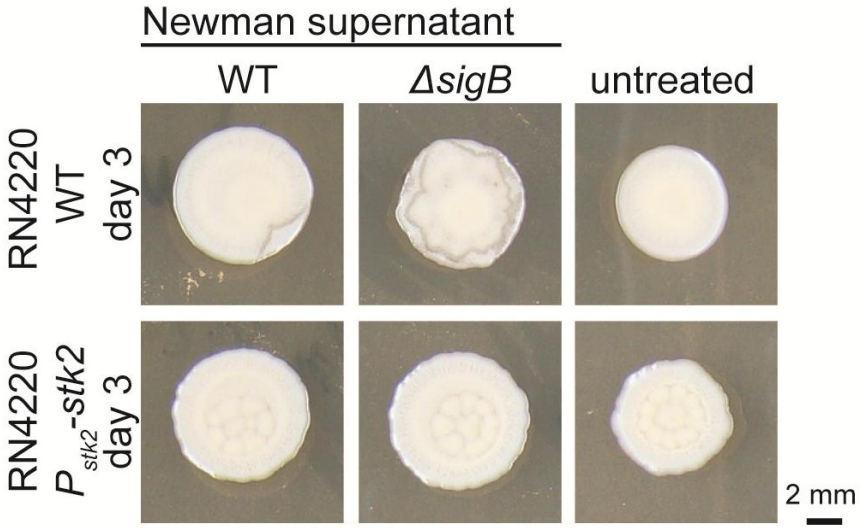

Fig. 40: Stk2 protects macrocolonies from lysis.

RN4220 macrocolonies are lysed by Newman culture supernatants (upper panel). RN4220 strain expressing Stk2 is non-susceptible to this lysis (lower panel). Macrocolonies were imaged after 3 days. Scale bar, 2 mm.

III. Discussion

III.1 The macrocolony biofilm model

The macrocolony model of *S. aureus* mimics magnesium-rich infection niches in the host (Garcia-Betancur et al., 2017, Koch et al., 2014b). The biodegradability of magnesium and magnesium alloys has created interest in a possible application of the materials as temporary implants (Waizy et al., 2013). Additionally, an antibacterial activity of magnesium-based materials against several clinically-relevant pathogens was reported (Feng et al., 2016, Li et al., 2014, Robinson et al., 2010, Xie and Yang, 2016). Nevertheless, the vulnerability of implants to biofilm-associated infections and the magnesium-dependent modulation of virulence factor expression in *S. aureus* pose hazards in this application (Garcia-Betancur et al., 2017, Jensen et al., 1999). As demonstrated in this thesis, the addition of magnesium salts to the culture medium strongly stimulates biofilm formation of *S. aureus* in two different model systems. Accordingly, magnesium implants prolong infections with *S. aureus* and *P. aeruginosa* in mice (Rahim et al., 2016). Biofilm-characteristic ECM material is found at the infection sites and the infections are highly resilient to systemic antibacterial therapy (Rahim et al., 2016). Consequently, the effect of magnesium on virulence, particularly biofilm formation, of *S. aureus* and other species must be considered in the development of biodegradable implants.

The macrocolony model provides an alternative approach to studying the processes related to bacterial biofilm formation. The structured colony surface of macrocolony aggregates is characteristic for this biofilm model. The formation of this morphology is dependent on the production of ECM (Branda et al., 2006, Ray et al., 2012, Serra and Hengge, 2017). The evaluation of biofilm formation differs between macrocolony aggregates and submerged biofilms. Submerged biofilms are commonly used in a quantitative assay, e.g. crystal violet assay (O'Toole, 2011, Stiefel et al., 2016). Macrocolonies are visually inspected for the colony morphology, optionally aided by microscopic approaches to study the cell morphology and matrix composition (Branda et al., 2004, Dietrich et al., 2008, Serra et al., 2013a). The

definition of the phenotypic categories presented in Fig. 11 was also based on visual inspection of the macrocolonies, but this approach is prone to inaccuracy due to the subjectivity of the operator. A computational analysis quantifying the complexity and growth kinetics of *B. subtilis* colonies was performed to overcome this shortcoming of the macrocolony biofilm model (Gingichashvili et al., 2017). This semi-quantitative approach has the potential to increase the objectivity of macrocolony characterization and the authors speculate that an extension of the method to other species including *S. aureus* is possible (Gingichashvili et al., 2017).

The ECM composition of *E. coli* and *Salmonella enterica* serovar Typhimurium macrocolonies has been studied by fluorescence microscopy. Both species produce the amyloid fiber curli and the polysaccharide cellulose in this model. Several fluorescent dyes – Thioflavin S, Calcofluor White, Pontamine fast scarlet 4b and Congo red – are established tools for the specific *in situ* detection of these components (Serra et al., 2013a, Serra et al., 2013b, Zogaj et al., 2003, Zogaj et al., 2001). The ECM of submerged *S. aureus* biofilms was characterized using confocal laser scanning microscopy (CLSM). PNAG can be stained by fluorophore-coupled wheat germ agglutinin which specifically binds to N-acetylglucosamines (Cerca et al., 2011, Oniciuc et al., 2016, Wright, 1984). Similarly, commercially available fluorescent dyes with DNA- or protein-binding properties can visualize the respective matrix components (Foulston et al., 2014, Oniciuc et al., 2016, Torres et al., 2016). It has not been demonstrated whether the above-mentioned fluorophores are also suitable for fluorescence microscopy of *S. aureus* macrocolonies or macrocolony cross-sections in a similar manner. Generally, fluorescence microscopy can be implemented in *S. aureus* macrocolony cross-sections. Using fluorescent reporter gene fusions, differential expression of biofilm-related genes depending on the spatial localization within the macrocolony was shown (Garcia-Betancur et al., 2017).

III.2 Identification of macrocolony biofilm regulators in a genome-wide screen

A central question of this thesis regards the factors required for the aggregation and surface structuring of macrocolonies. The processes that drive the characteristic surface structuring are poorly understood in *S. aureus*. PSM are important factors that shape the 3D structure of submerged biofilms (Periasamy et al., 2012). Similarly, PSM contribute to the structure of macrocolony biofilms. Their deletion decreases yet not eradicates the surface structure (Garcia-Betancur et al., 2017).

In this thesis, I used an asset of the macrocolony biofilm model, its scalability to screen a large number of clones or a library, to study factors that contribute to the surface structure. Accordingly, novel macrocolony morphologies were identified and described by screening a genome-wide mapped transposon library (Fey et al., 2013). The high open reading frame (ORF) coverage of the library of 76% enabled a comprehensive assessment of the majority of genes of *S. aureus* (Fey et al., 2013). This is in contrast with a previous screen in *B. subtilis*, which used a library containing only non-characterized genes and revealed six genes with different roles in the community development (Branda et al., 2004). In our study, only three of the 28 candidates identified as novel factors in biofilm formation in the screen of the NTML have unknown gene functions: SAUSA300_0538, SAUSA300_1008, SAUSA300_1118. Hence, most of the candidates were identified in a context that differs from the function they were originally assigned.

These results emphasize the importance of characterizing gene functions in diverse environmental conditions as their effect can be multifactorial. In other words, the phenotype caused by a gene must be assessed in consideration of the environmental conditions (Darwin, 1859, Griffiths et al., 2000). Different environmental stimuli on populations of the same genotype can manifest in phenotypic heterogeneity (Ackermann, 2015). Besides the environmental influence, moonlighting proteins that engage in completely unrelated molecular pathways or functions, e.g. glycolytic enzymes moonlight to the ECM of biofilms,

are a possible reason for the involvement of proteins in several functions (Foulston et al., 2014, Henderson and Martin, 2011).

III.3 Novel pathways required for the formation of macrocolony biofilms

The majority of the candidates identified in the screen of the NTML based on their macrocolony morphology are involved in carbohydrate and nucleotide metabolism. Thereby, this work identifies a novel link between macrocolony aggregation and basic metabolic processes of *S. aureus*.

Transposon mutants deficient in **nucleotide metabolism** were the most abundant group identified in the NTML screen. When purines can not be recycled via the energy-conserving salvage pathway, bacteria perform *de novo* purine biosynthesis (Switzer et al., 2002). The constant supply with purines is important in *S. aureus* as indicated by the virulence- and growth-defect of purine-depleted mutants (Lan et al., 2010, Mei et al., 1997). In this work, *ΔpurK* was not attenuated in virulence in a non-vertebrate infection model. An earlier study that linked purine biosynthesis to virulence used a *purA* mutant (Lan et al., 2010). This strain has defects in *de novo* purine synthesis as well as purine recycling, whereas the salvage pathway is functional in *ΔpurK*. Additionally, the relative effect of the gene deletion depends on the infection site, e.g. PurK-deficient strains can grow in abscesses but are incapable of growing in chronic wounds (Ibberson et al., 2017). Chronic wounds are associated with a biofilm-like lifestyle, but it was not assessed whether the essentiality of *purK* in chronic wounds was related to alterations in biofilm formation (Akiyama et al., 1996, Ibberson et al., 2017). An effect of purine biosynthesis on biofilm formation has been reported in *S. epidermidis* as the transposon-disruption of the homologue of the repressor *purR* results in a biofilm-negative strain (Knobloch et al., 2003). The mechanism of this biofilm defect is obscure, but it is speculated that a σ^B-binding site in the repressor gene is involved in the biofilm defect (Knobloch et al., 2003, Mack et al., 2007). For *ΔpurK* of *S. aureus*, it was demonstrated in this work that the increase of biofilm formation is not dependent on the action of σ^B. In *Pseudomonas fluorescens* and *Burkholderia*-species in the insect gut, purine

biosynthesis is required for biofilm formation (Kim et al., 2014, Yoshioka and Newell, 2016). The mechanism by which purine biosynthesis affects biofilm formation differs between the species. In *P. fluorescens*, purine biosynthesis-defective cells attached to surfaces less efficiently and the cell size was reduced, thus reducing the biofilm biomass (Yoshioka and Newell, 2016). It is not known whether defects in purine biosynthesis affect the cell size in *S. aureus*. In *Burkholderia*, purine-deficient mutants have lower levels of the second messenger c-di-GMP, a positive regulator of biofilm formation, due to restricted availability of purines (Kim et al., 2014). C-di-GMP is important for the switch between motile and sessile lifestyles in many gram-negative species including *E. coli*, *P. aeruginosa* and *S. enterica* (Hengge, 2009, Jenal and Malone, 2006, Romling and Balsalobre, 2012). The role of c-di-GMP for biofilm formation in *S. aureus* is ambiguous. Studies have presented conflicting data whether c-di-GMP affects biofilm formation positively or negatively (Holland et al., 2008, Ishihara et al., 2009, Karaolis et al., 2005).

Notably, it was demonstrated that the effect of purine biosynthesis defects on *S. aureus* macrocolony aggregation is not uniform. Depending on the gene in which the transposon is inserted, biofilm formation is increased, decreased or not altered. A possible polar effect of the transposon insertion is conceivable as the genes, whose disruption causes no effect, cluster towards the end of the operon. The genes in the *pur* operon are not in the same order as the enzyme activity in the purine synthesis pathway occurs (Hartman and Buchanan, 1959, Zhang et al., 2008). The diversity of phenotypes of purine biosynthesis-deficient mutants could be explained by moonlighting functions of one or several of the proteins. PurK was identified as an adhesive polypeptide with binding-capacity for human fibrinogen but follow-up studies to determine if the moonlighting function is an artifact due to the protein expression in a heterologous host are not available (Kylvälä et al., 2011). However, the restoration of a WT-like macrocolony phenotype by metabolic supplementation argues against a moonlighting function of PurK in this context.

Among the identified genes involved in **carbohydrate metabolism** is the gene coding for the enzyme PckA, which catalyzes the initial step of the glucose-generating pathway gluconeogenesis (Anderson and Wood, 1969). Glucose is commonly supplemented to biofilm-inducing growth media in *S. aureus* due to its positive effect on biofilm formation, but gluconeogenesis *per se* has not been suggested to have this effect on biofilm formation (Lim et al., 2004, Waldrop et al., 2014). Interestingly, the *pckA* homologue in *Saccharomyces cerevisiae* is >500-fold upregulated during the attachment phase of the biofilm compared to planktonic cells (Li et al., 2015). This upregulation co-occurs with the repression of glycolysis. Thus, carbohydrate synthesis is linked with early stages of biofilm formation in *S. cerevisiae* (Li et al., 2015).

It has been reported that the *in vivo* expression of *S. aureus pckA* varies depending on the infection site. The gene is repressed during the early niche adaptation in a murine pneumonia model (Chaffin et al., 2012). In contrast, during acute and chronic osteomyelitis, *pckA* expression is higher than *in vitro* (Szafranska et al., 2014). The dynamic regulation of *pckA* expression represents an example of adaptation to environmental conditions. Despite the dynamic regulation of *pckA*, the mutant is not attenuated in virulence in a murine infection model, which is in accordance with the results of the non-vertebrate infection model in this work (Vitko et al., 2015). In physiological contexts, the importance of gluconeogenesis can be glucose-independent as intermediates of the pathway are also processed to different products. The intermediate fructose-6-phosphate, which occurs in gluconeogenesis as well as glycolysis, can be metabolized to be incorporated into PNAG, peptidoglycan, capsule polysaccharides and teichoic acids (Sadykov et al., 2010). Interestingly, fructose-6-phosphate is synthesized by the enzyme Fbp, whose mutant was the other candidate classified as category 2 besides *ΩpckA*. It can be hypothesized that the macrocolony biofilm defect of *ΩpckA* and *Ωfbp* occurs through a related mode of action. In contrast, the mutants involved in carbohydrate metabolism via the TCA cycle (*ΩodhB, ΩgltA, ΩfumC, Ωmqo*) were categorized into category 1 as they are biofilm deficient but lack the characteristic flatness of category 2 candidates.

Besides genes involved in metabolic processes, the results from this work show a dominant role of the **response regulator SrrA**, which is part of the redox-sensitive TCS SrrAB, for structuring of the macrocolony surface. *S. aureus* contains 16 TCS, of which only one (WalKR) is essential, to control gene expression in response to environmental cues (Cheung et al., 2004, Dubrac et al., 2007, Martin et al., 1999). The activity of the TCS SrrAB is driven by the state of the respiratory chain (Kinkel et al., 2013, Mashruwala et al., 2017a, Mashruwala et al., 2017b). The *in vivo* data in this thesis confirms earlier reports that SrrAB is required for virulence in *S*. *aureus* (Pragman et al., 2007a, Pragman et al., 2004). SrrAB is embedded in the signal transduction network of *S. aureus* and affects *agr* expression (Pragman et al., 2004, Yarwood et al., 2001). The repression of Agr could not be seen in the hemolysis assay in this work but it must be considered that Agr is repressed to low levels in a σ^B-dependent manner due to the addition of magnesium to the growth medium (Garcia-Betancur et al., 2017). In this thesis, a novel epistatic relationship between *srrA* and *sigB* was identified. The epistasis becomes apparent in the hemolysis assay where *ΔsigB* conventionally shows increased hemolysis. This phenotype is absent in the *ΔsrrA ΔsigB* genotype, which reproduces the hemolytic activity of WT and *ΔsrrA*. As a consequence, we speculated that SrrA acts as a modulator of the Agr-σ^B signaling cascade, which controls macrocolony biofilm formation in *S. aureus*.

The spatial distribution of SrrAB activation within the macrocolony is unknown. Oxygen depleted zones are detected in biofilms beyond a depth of 40 – 100 µm (de Beer et al., 1994, Kuhl et al., 2007). Based on these values, vast hypoxic zones can be expected in *S. aureus* macrocolonies, which reach a thickness of 300 – 400 µm (Garcia-Betancur et al., 2017). The use of fluorescent reporter gene fusions is only partially suitable to determine SrrAB activity. The autoinductivity of SrrAB makes fluorescent reporters of the *srrAB* promoter activity an approximation of the TCS' activity (Pragman et al., 2004). Exact determination of the activity should assess the phosphorylation state of the response regulator. Additionally, it must be considered that *srrA* can be transcribed independently of *srrB* (Yarwood et al., 2001). The independent transcription of the genes of the *srrAB* operon is remarkable in the context that

only *ΩsrrA* had alterations of macrocolony morphology but *ΩsrrB* did not. The complementation of the WT phenotype with *srrA* rules out polar effects of the gene deletion. The typical signal transduction relay of a TCS includes a HK and its cognate RR (Stock et al., 2000). Cross-talk refers to the phenomenon that a HK can phosphorylate a non-cognate RR (Laub and Goulian, 2007, Wanner, 1992). In *E. coli*, a systematic search between the 30+ TCS showed possible cross-talk among 3% of all possible HK-RR combinations (Yamamoto et al., 2005).

Hitherto, two examples of cross-talk, both involving the TCS GraRS, have been identified in *S*. *aureus*. The HK GraS cross-talks with ArlR and the RR GraR is cross-regulated by the serine-threonine kinase Stk1 (Fridman et al., 2013, Villanueva et al., 2018). The former example was described in a study that used a systematic approach to identify interactions between response regulators and non-cognate histidine kinases to improve the understanding of signal integration in *S. aureus* (Villanueva et al., 2018). There were no indications that SrrA was involved in cross-talk, hence excluding this phenomenon as an explanation for the divergence between the *ΩsrrA* and *ΩsrrB* macrocolony phenotypes.

SrraAB serves as a switch altering gene expression between aerobic and anaerobic conditions in *S*. *aureus* and also in *S*. *epidermidis*, whose SrrAB is 90% identical to *S*. *aureus* (Kinkel et al., 2013, Wu et al., 2015). Interestingly, among the repressed genes in *ΔsrrAB* are *ctaB* and *qoxA* in *S*. *aureus* and *ctaAB* and *qoxA* in *S*. *epidermidis* (Kinkel et al., 2013, Wu et al., 2015). The mutants of these genes, which are required for the maintenance of the electron transport chain, were also detected in the screen of the NTML and classified as category 1 because they formed macrocolonies with normal thickness that did not develop wrinkles on the surface. Notably, a growth defect after 10 h was found for *ΩctaB* and *ΩqoxA,* but not for *ΩsrrA* and *ΩctaA*. Due to the similar macrocolony morphologies of *ΩsrrA* and mutants, which are defective in the electron transport chain, it can be speculated that the decreased expression of genes in the electron transport chain contributes to the biofilm-defective phenotype of *ΔsrrA* in the macrocolony model.

Beyond the factors that were characterized in Fig. 18-Fig. 27, the screen identified 25 further factors that affected macrocolony morphology (see Table 1). The mode of action of most of these factors remains obscure. For 2 candidates, namely *mpfA* and *fakA*, existing literature provides potential insights into the mode of action. MpfA was recently identified as a membrane-bound transporter involved in magnesium-tolerance (Armitano et al., 2016). While the *mpfA* mutant does not have a growth-deficit in TSB, mutants of this gene are sensitive to magnesium concentrations as low as 10 mM (Armitano et al., 2016). This is 10-fold lower than the $MgCl_2$-concentration found in TSBMg and almost 80-fold lower than the normal magnesium-tolerance of *S. aureus* (Cebrian et al., 2014, Koch et al., 2014b). Therefore, the biofilm-deficient phenotype of *ΩmpfA* in the macrocolony model is possibly related to the magnesium-sensitivity of the strain. Using the same transposon library used in this thesis, *fakA* has been identified as a factor in biofilm formation (Sabirova et al., 2015). As *ΩfakA* produces increased levels of biofilms in a dynamic flow model in BHI medium, it can be speculated that the effect of FakA on biofilm formation is not solely dependent on magnesium-rich conditions as found in the macrocolony model (Sabirova et al., 2015).

Among the identified candidates, *ΩclpP* was unique in showing divergent effects of transposon insertion on quantitative biofilm assays and the macrocolony assay. The crystal violet assay of submerged biofilms in multiwell polystyrene plates is the standard approach for quantification of biofilm biomass and evaluation of biofilm eradication strategies (Stiefel et al., 2016). The macrocolony model of *S. aureus* was validated by demonstrating that alterations of the cell wall structure had similar effects on macrocolony aggregation and submerged biofilms in magnesium-rich conditions (Garcia-Betancur et al., 2017). Nevertheless, the *clpP*-mutant showed decreased wrinkling in the macrocolony assay but increased formation of submerged biofilms regardless of the growth medium. The effect of the protease ClpP on biofilm formation is heavily dependent on environmental conditions. In accordance with the results found in this thesis, *ΔclpP* was described as an overproducer of submerged biofilms in TSB-NaCl-glu and TSB + 0.5% glucose (Atwood et al., 2015, Liu et al., 2017b). This effect is niche-dependent as biofilm formation in TSB and on plasma-coated

surfaces does not differ between WT and *ΔclpP* (Atwood et al., 2015). Furthermore, the deletion of *clpP* has a positive effect on biofilm formation by decreasing *agr* activity (Frees et al., 2003, Liu et al., 2017b). In the macrocolony model, *agr* activity is already very low due to σ^B-dependent repression (Garcia-Betancur et al., 2017). This example highlights the plasticity of biofilm formation regulation in response to the environmental niche.

Interestingly, while the screen described several novel macrocolony phenotypes by genes primarily unrelated to biofilm formation, it did not yield any of the around 30 surface adhesion factors to be important for macrocolony formation (Clarke and Foster, 2006, Heilmann, 2011). There are limitations to the screen, which could cause this surprising finding. Firstly, the surface adhesion factors are partially redundant as there are examples of multiple adhesins binding to the same host target (Foster et al., 2014, Zapotoczna et al., 2016). However, the NTML only contains mutants with a single transposon insertion in the genome (Fey et al., 2013). Therefore, the loss of function of individual adhesins can be masked by a functionally redundant adhesin. Secondly, surface adhesion factors are required for *S. aureus* to initiate binding to host or artificial surfaces under shear stress but the macrocolony model does not generate shear stress on the biofilm (George et al., 2006). An adaptation of the experimental conditions to biofilms formed under dynamic media flow could aid to determine, which role surface adhesins play for biofilm formation in TSBMg. Thirdly, the surface adhesins mediate the formation of submerged biofilms by intercellular adhesion as well as adhesion to the polystyrene wells (Cucarella et al., 2001, Schroeder et al., 2009). Characterization of macrocolony biofilms is focused on polymeric ECM components that shape the colony morphology instead of attachment-mediating factors (Serra and Hengge, 2017). An example of a surface-attachment factor that affects the colony biofilm morphology is found in *Vibrio cholerae*. The biofilm-associated protein 1 (Bap1) is a secreted protein that localizes at the biofilm-surface interface and mediates attachment to the substratum (Absalon et al., 2011, Berk et al., 2012). The wrinkling of rugose *V. cholerae* colony biofilms is decreased in absence of Bap1 (Fong and Yildiz, 2007). Further studies are warranted to

understand the forces that attach macrocolony biofilms to the substrate and which adhesins are required in this context.

In conclusion, numerous novel macrocolony phenotypes were described in section II.1 of this thesis. Thereby, purine biosynthesis and gluconeogenesis were identified as vital metabolic processes for macrocolony biofilm formation in *S. aureus*. Additionally, a regulatory role for the oxygen-dependent response regulator SrrA was found and its action was linked to the established macrocolony regulators Agr and σ^B. These findings improve the understanding of biofilm formation-like processes in magnesium-rich infection niches. Furthermore, it will be interesting to study the mode of action of the novel regulators as well as whether they affect macrocolony-related processes, in particular the *agr*-dependent phenotypic differentiation (Garcia-Betancur et al., 2017).

III.4 Phage-mediated competition in mixed communities

Bacterial interactions in a clinical isolate of *S. aureus* can cause genetic diversification and the emergence of new phenotypic traits in the macrocolony biofilm model (Koch et al., 2014b). In section II.2 of this thesis, the macrocolony biofilm model is used to study the interactions occurring between different *S. aureus* strains in the multicellular aggregates as the interstrain competition results in macroscopically visible lytic processes.

The collected data suggests an involvement of prophages in the induction and maintenance of biofilm-specific lysis. This hypothesis is supported by the sedimentation of the active particle to the lowest fraction of the sucrose gradient. Furthermore, the lysis could be recreated in a system with artificially transduced strains and immunity against phage infections prevented lysis of the macrocolonies.

The observed limitation of phage-mediated lysis to biofilm-inducing conditions is remarkable, because the ECM poses a barrier to phage infections (Gutierrez et al., 2016, Vidakovic et al., 2017). This barrier is also relevant as phage therapy is commonly suggested as a means of biofilm removal (Alves et al., 2014, Gutierrez et al., 2015, Kelly et al., 2012, Negut et al.,

2016, Pires et al., 2017). On the other hand, the ECM can cause entrapment of phage particles within the biofilm and locally increase their concentration (Briandet et al., 2008, Doolittle et al., 1996, Lacroix-Gueu et al., 2005).

The dynamics between biofilms and phages vary depending on the stage of biofilm formation (Fernandez et al., 2018). It has been argued that maturation of the biofilm improves the protection from phages and that biofilm-bound cells are still vulnerable to phage infections shortly after adhesion (Abedon, 2016). In accordance with the plasticity of the phage-biofilm interactions, the onset of lysis in the mixed communities occurs within the first 24 h of macrocolony development.

It is conceivable that the environment in macrocolony aggregates alters the nature of the interstrain interactions. The observation that USA300 is only susceptible to Newman-triggered lysis under biofilm-inducing conditions is a good example for the niche-dependency of interactions. The growth in multicellular aggregates on TSBMg agar alters several aspects of cellular physiology by changing gene expression profiles (Garcia-Betancur et al., 2017). Potential changes in the expression of genes involved in phage defense could be causative for the susceptibility to lysis. Additionally, the induction of macrocolony aggregation is based on reduced ionic charges of WTA, which are also surface receptors for phages (Xia et al., 2011). Thereby, the addition of magnesium to the growth medium to induce biofilm formation could alter the phage infection efficiency. Contrariwise, phage exposure also has diverse effects on physiological traits of staphylococcal cells in biofilm communities including the repression of nucleotide biosynthesis, DNA replication and aerobic respiration as well as the activation of TA biosynthesis, capsule biosynthesis and the stringent response (Fernandez et al., 2017).

Although the activation of one or several prophages seems the most probable cause of lysis, the lysis mechanism could not be elucidated. Specifically, it was found that the biofilm-specific lysis of USA300 is independent of the SOS response. Hence, its mode of action differs from the “remote-controlled” manner described for the interspecies competition assay

of *S*. *pneumoniae* and *S*. *aureus* (Selva et al., 2009). Since the lysis-triggering particle could not be purified, a competition mechanism different from phage-induced lysis must be considered.

The strains used in the experiments (Newman, USA300, RN450) all produce AIP type-I, hence ruling out interference of the QS system (Baba et al., 2008, Diep et al., 2006, Ji et al., 1997, Traber and Novick, 2006). In mixed-community macrocolonies, the competing strains segregate into distinct sectors. A similar segregation was found in simulations of co-cultured toxin-producing and toxin-susceptible strains (Nadell et al., 2016, Weber et al., 2014). The segregation of competing strains allows for the establishment of a stable coexistence. Hence, phage- and toxin-mediated competitions have similar effects on the strain distribution during community development. An analogy between phage- and toxin-mediated predation was previously noted:

> *"In other words, lysogenic cells may use viral particles in a way similar to the use of toxins to kill non-lysogenic susceptible strains, thus possessing a fitness advantage over them*" (Gama et al., 2013)

The toxin-like and phage-like characteristics of the lytic particle could be consolidated in a model in which a lysis-triggering toxin is encoded on prophage-contained DNA.

III.5 Biological significance of intraspecies competition

The activity of phages as biological weapons against competitors is documented in diverse bacterial species and environments (Bossi et al., 2003, Gama et al., 2013, Haaber et al., 2016, Joo et al., 2006). Phage predation is limited to closely related species by the phage specificity that is determined by phage- and host-associated factors (Hyman and Abedon, 2010, Koskella and Meaden, 2013). The benefits of phage-mediated predation for the phage-releasing lysogenic strain include (i) killing of competitors, (ii) subsequent consumption of nutrients released by lytic cells and (iii) genetic diversification by uptake of released DNA

(Harrison and Brockhurst, 2017, Resch et al., 2005a, Weitz and Wilhelm, 2012). The latter process is termed “autotransduction” and defined as:

> *“[…] spontaneously released phages from a subpopulation of lysogenic bacteria propagate on phage-susceptible, co-cultured target bacteria and, subsequently, with high frequencies, transfer DNA from the lysed cells back to the remaining lysogenic cell population*” (Haaber et al., 2016)

Autotransduction occurs in liquid medium as well as *in vivo* in *G mellonella* (Haaber et al., 2016). The mixed-community set-up comprises the lysogenic staphylococcal strains Newman and USA300 that harbor four and two prophages, respectively (Bae et al., 2006, Diep et al., 2006). Theoretically, in this set-up the Newman strain could benefit from autotransduction because the lytic USA300 strain is a highly virulent MRSA isolate carrying antibiotic resistance genes. However, evolution is not a directed process and such processes do not occur simply because they would provide a biological benefit (Lenormand et al., 2009). The isolation of Newman clones harboring newly acquired antibiotic resistances or virulence factors from mixed communities could demonstrate whether this strain indeed benefits from the interstrain competition by genetic diversification.

Additionally, phage-predation has community-wide effects by evolutionary and non-evolutionary adaptations that are specific to biofilm-associated communities (Hosseinidoust et al., 2013). The former effect is evidenced by *P. fluorescens*, which produces more ECM after co-evolution with a lytic phage (Scanlan and Buckling, 2012). The latter effect is exemplified by the contribution that eDNA, which is released from lysed cells, makes to the ECM under biofilm forming conditions in *S. aureus* (Fernandez et al., 2017). Future studies are required to determine the quantitative effects and the long-term impact of interstrain competition on biofilm communities of *S. aureus*.

In conclusion, the occurrence of interstrain competition between *S. aureus* isolates, which manifested as lytic sectors in a macrocolony biofilm model, was described in section II.2 of

this thesis. Analysis of the underlying processes exposed an involvement of bacteriophages in the induction of lysis. It is tempting to speculate whether the processes that regulate biofilm-specific lysis by interstrain competition in macrocolony biofilms could be engineered into a biofilm-specific antimicrobial strategy active against USA300 strains, which are the predominant MRSA lineage in the United States (Talan et al., 2011, Tenover and Goering, 2009).

IV. Material and Methods

IV.1 Chemicals and materials

IV.1.1 Common chemicals and consumables

Common chemicals were purchased from the following suppliers: AppliChem (Darmstadt), Merck (Darmstadt), Roth (Karlsruhe) and Sigma-Aldrich (Hamburg). Molecular biological studies were performed with material purchased from Macherey-Nagel (Düren), Qiagen (Hilden) and New England Biolabs (Frankfurt a.M.). Single-use consumables were purchased from local distributors and autoclaved before use when required.

IV.1.2 Enzymes and specialized chemicals

Enzymes and specialized chemicals that were used in this work are listed in Table 3.

Table 3: Enzymes and specialized chemicals used in this work

Compound	Manufacturer
1 kb DNA Ladder	New England Biolabs
Ambicin® L (recombinant lysostaphin)	Ambi Products
Crystal Violet	Roth
DNase I	New England Biolabs
Expand™ Long Template PCR System	Sigma-Aldrich
Orange G	Omikron
Phusion Polymerase	New England Biolabs
Proteinase K	Merck
Restriction Enzymes	New England Biolabs
RNase A	Sigma-Aldrich
Roti®-Phenol (pH 7.5 – 8)	Roth
Roti®-Phenol/Chloroform/Iso amyl alcohol	Roth
Sheep erythrocytes in Alsever's solution	Elocin Lab
Taq Polymerase	New England Biolabs

IV.1.3 Buffers

Lysis buffer	20 mM Tris
	10 mM EDTA
	pH 7.5
Sucrose Gradient Buffer	25 mM MES
	150 mM NaCl
	pH 6.5
50x TAE buffer	2 M Tris
	1 M Acetic acid
	20 mM EDTA
	pH 8.0
3x DNA loading buffer	100 µM Orange G
	30% (v/v) glycerol
1x PBS	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	1.8 mM KH_2PO_4
	pH 7.4
Coomassie staining solution	0.5 g Coomassie brilliant blue R-250
	500 ml dH_2O
	400 ml methanol
	100 ml acetic acid
Destaining solution	700 ml dH_2O
	200 ml methanol
	100 ml acetic acid

4x Laemmli buffer	250 mM Tris-HCl
	40% (v/v) glycerol
	4% SDS
	0.02% (w/v) bromophenol blue
	10% (v/v) β-mercaptoethanol
	pH 6.8
SDS Running buffer	25 mM Tris
	192 mM glycine
	0.1% (w/v) SDS
	pH 8.3

IV.1.4 Laboratory equipment

Laboratory equipment that was used in this work is listed in Table 4.

Table 4: Laboratory equipment used in this work

Laboratory Equipment	Manufacturer
Axiocam Color 412-312	Zeiss
Centrifuge Avanti J-26 XP	Beckman Coulter
Cooling Centrifuge 5427R	Eppendorf
Cybershot DSC-H50 digital camera	Sony
Gel iX Imager	Intas Science Imaging
Gradient Station	BioComp Instruments
InfiniteF200 Pro microtiter plate reader	Tecan
inoLab pH 720	WTW
Micropulser Electroporator	Bio-Rad
Multifuge X3R	Thermo Fisher
Nanodrop 1000	Thermo Fisher
PerfectBlue™ Gel System	Peqlab
PowerPac 300	Bio-Rad

Laboratory Equipment	Manufacturer
SMZ1500 stereomicroscope	Nikon
Sonication probe: MS73	Bandelin
Sonopuls	Bandelin
Spectrometer Ultrospec 3100pro	Amersham Biosciences
Swinging bucket rotor SW 40.Ti	Beckman Coulter
Thermocycler T3	Biometra
Ultracentrifuge Optima™ L-100XP	Beckman Coulter

IV.1.5 Software

Software that was used in this work is listed in Table 5.

Table 5: Software used in this work

Software	Manufacturer
Axio Vision	Zeiss
Clone Manager Professional	Sci-Ed
EndNote	Clarivate Analytics
Ilustrator	Adobe
ImageJ	Wayne Rasband
Marvin Sketch	Chemaxon
Office 365	Microsoft
Prism	GraphPad

IV.2 Microbiology

IV.2.1 Strains, media and culture conditions

The clinical isolates *S. aureus* Newman (Duthie and Lorenz, 1952) and USA300_TCH1516 (Gonzalez et al., 2005) were used for macrocolony aggregates. The NTML is in the plasmid-cured USA300-JE2 background (Fey et al., 2013). *Escherichia coli* strain DH5α (Reusch et al., 1986) and *S. aureus* RN4220 (Kreiswirth et al., 1983) were used for cloning.

E. coli was propagated in lysogeny broth (LB) medium. *S. aureus* was propagated in tryptic soy broth (TSB) medium or, for infection of larvae of the wax moth *Galleria mellonella*, in brain-heart-infusion (BHI) medium. Unless indicated otherwise, cells were grown with 220 rpm shaking at 37°C. Culture volumes were at most 1/10 of the flask volume. For agar plates, the medium was autoclaved with 1.5% (w/v) agar. For macrocolony assays, TSB was supplemented with 100 mM $MgCl_2$ (TSBMg). When required, selective media were prepared by adding antibiotics at the concentrations indicated in Table 6.

IV.2.2 Glycerol stocks

Glycerol stocks were prepared for permanent storage of strains at -80°C. Cells were grown on plates overnight, scraped off and resuspended in 1 ml medium containing 20% (v/v) glycerol.

IV.2.3 Antibiotics and media supplements

Antibiotics used in this work are listed in Table 6.

Table 6: Antibiotics used in this work

Compound	Supplier	Final Concentration
ampicillin	Roth	100 µg/ml
erythromycin	Roth	2 µg/ml (RN4220, Newman, USA300-JE2) 125 µg/ml (USA300_TCH1516)
tetracycline	AppliChem	15 µg/ml
spectinomycin	Sigma-Aldrich	600 µg/ml

Media supplements used in this work are listed in Table 7.

Table 7: Media supplements used in this work

Compound	Supplier	Final Concentration
Calcium chloride	Roth	5 mM (phage transduction) 50 mM (biofilm assay)
Citrate	Roth	10 mM
Glucose	Roth	0.5% (w/v) = 27.8 mM
Inosin monophosphate	Sigma-Aldrich	500 mM

Compound	Supplier	Final Concentration
Magnesium chloride	Roth	100 mM
Magnesium sulfate	Merck	100 mM
Manganese chloride	Roth	20 mM
Potassium chloride	Roth	100 mM
Sodium chloride	Roth	100 – 517 mM
Sodium pyruvate	Sigma-Aldrich	0.5% (w/v) = 45.5 mM
X-Gal	Roth	50 - 100 µg/ml = 122.5 - 245 µM

IV.2.4 Media formulations

LB (Lennox)	10 g/l tryptone
	5 g/l yeast extract
	5 g/l NaCl
TSB	30 g/l commercial formulation (BD)
BHI	37 g/l commercial formulation (Sigma-Aldrich)

IV.3 Microbiology techniques

IV.3.1 Biofilm assays

For macrocolony assays, strains were streaked from glycerol stocks on TSB agar plates and incubated overnight. Cells were collected from the plate and resuspended in 50 µl TSB. Two µl of the suspension were spotted on TSBMg agar plates and statically incubated at 37°C for up to five days. To test resistance to dispersal, the surface of the macrocolonies was carefully touched with a blunt object (e.g. pipette tip or scalpel). The object was carefully moved sideways while maintaining gentle pressure on the macrocolony until fragmentation was observed.

Quantitative biofilm assays were performed based on a procotol by O'Toole and Kolter (O'Toole and Kolter, 1998). Briefly, strains were grown overnight in 2 ml TSB. The OD_{600} was measured and precultures were started with an OD_{600} = 0.05 in 2 ml TSB. After 4-6 h, the precultures were used to set submerged biofilms in flat-bottom 24-well plates with a normalized OD_{600} = 0.05. Submerged biofilms were incubated statically in a volume of 1 ml growth medium per well for up to 48h. To quantify biofilm formation, crystal violet staining was performed. The medium was carefully discarded from the multiwell plates and wells were washed twice with 1x PBS. Biofilms were fixed at 65°C for 30 min. To stain the biofilm material, 500 µl crystal violet solution (0.1% (w/v)) was added and incubated for 5 min. The staining solution was discarded and plates were washed at least three times with H_2O until no more dye was seen in the washing solution. For quantification, 500 µl acetic acid (33% (v/v)) was used to solubilize the dye. Absorbance of the solution was measured at 595 nm in a spectrophotometer (Ultrospec 3100 Pro, Amersham Biosciences) or InfiniteF200 Pro microtiter plate reader (Tecan) at an appropriate dilution. The crystal violet assay of the NTML candidates was performed in 96-well plates. To account for the smaller volume of the wells, 200 µl growth medium and 100 µl of the crystal violet solution and acetic acid for solubilization were used in the assay.

IV.3.2 Screen of the NTML

All 1,920 mutants of the NTML (Fey et al., 2013), which is stored in 96-well plates at -80°C, were screened for their macrocolony phenotype. Cells were transferred from the glycerol stocks onto TSB plates and incubated at 37°C overnight. Material from the colonies was transferred onto TSBMg plates and incubated at 37°C for 5 days. The macrocolonies were inspected visually under the stereomicroscope every 24 h. The thickness and structuring of the surface was evaluated to determine whether macrocolony phenotypes differed from WT and to assign corresponding transposon mutants to a phenotypic category.

IV.3.3 Growth curves

Growth curves of the candidates identified in the screen of the NTML were performed using an InfiniteF200 Pro microtiter plate reader (Tecan). Precultures of all strains were grown ON in 2 ml TSB and diluted to an OD_{600} = 0.05. Growth was measured in 96-well plates with 150 µl per well for 10 h in 30 min intervals.

Growth assays of USA300_TCH1516 WT and *ΔpckA* in different media were performed manually in culture flasks. Precultures of the strains were grown ON in 2 ml TSB and diluted to 20 ml of the respective medium with an OD_{600} = 0.05. Samples were taken every 2 h for 8 h and the OD_{600} was measured by photometer.

IV.3.4 IMP supplementation

Macrocolonies of the *ΔpurK* strain were supplemented with inosin monophosphate (IMP) to metabolically complement the purine biosynthesis defect. 10 µl 500 mM IMP were spotted onto TSBMg plates and dried for 10 min. Suspensions of bacterial cells were spotted onto the same spot as described in the section IV.3.1.

IV.3.5 Competition experiments

The relative fitness of *ΔpckA* was tested in a competition experiment with the WT. Both strains were grown ON in TSB. The OD_{600} was measured and each strain was added at an OD_{600} = 0.025 to 20 ml TSB (final OD_{600} = 0.05). The culture was incubated at 37°C and 1 ml samples were taken at the indicated times. Serial dilutions in 1x PBS were plated on TSB and selection plates with tetracycline. The plates were incubated for 24 h and colony forming units (CFU) were counted. The share of WT was determined by the difference of total CFU and CFU on selection plates.

IV.3.6 Staphyloxanthin extraction

Macrocolonies were grown on TSBMg prior to staphyloxanthin extraction. After the indicated incubation time, macrocolonies were harvested and suspended in 1 ml PBS each. The cells were collected by centrifugation at 9.400 x g for 1 min and thoroughly resuspended in 1 ml

PBS. The OD_{600} was determined to normalize the extracted staphyloxanthin to the cell density. After collecting the cells again, they were resuspended in 250 µl MeOH and incubated at 55°C for 3 min. The supernatant was collected after centrifugation. This step was repeated twice. The pooled supernatants were centrifuged to remove remaining cellular debris. The volume was adjusted to 1 ml with MeOH and absorption at 463 nm was measured (OD_{463}). Finally, the ratio of OD_{463} / OD_{600} was calculated to determine the relative staphyloxanthin production.

IV.3.7 Hemolysis assay

Hemolysis of USA300_TCH1516 WT and mutants was assessed in the supernatant of submerged biofilms in different media. The biofilm samples were prepared as described in the section IV.3.1. After 24 h, instead of removing the medium, 1 ml 4% (v/v) sheep erythrocytes was added to the wells and incubated at 37°C for 4 h. The multiwell plate was centrifuged at 500 x g for 15 min to pellet bacteria and unlyzed erythrocytes. The OD_{405} of the supernatant was measured with a photometer to quantify hemolysis.

IV.3.8 Mixed communities

Mixed communities were grown with Newman *ΔsigB* and USA300_TCH1516 WT unless indicated otherwise. Both strains were grown on TSB overnight and the cells were resuspended in 50 µl TSB. The strains were mixed at varying ratios (1:1 to 1:10) and 2 µl were spotted on TSBMg plates. The macrocolonies were always imaged in the same orientation to track the development of the sectors.

IV.3.9 Supernatant assay

Cell-free supernatant was used to characterize the lysing agent in the macrocolonies. Macrocolonies of the lytic strain were spotted on TSBMg as described in the section IV.3.1. Cultures of the strain to be tested for lysis potential were grown in 2 ml TSB for 6 h. The cells were removed by centrifugation at 10.000 x g for 5 min and subsequent filtration (0.2 µm). Eight hours after spotting the macrocolonies, 3-5 µl of the sterile supernatant were carefully dripped onto the colony. The occurrence of lysis was assessed after 3 days.

Proteins in the supernatant were digested by 150 µg/ml proteinase K at the indicated temperatures for 20 min. To exclude an effect of the temperature on the supernatant, a heat-control without the enzyme was also performed.

IV.3.10 Quantification of phage release in macrocolonies

The release of phages in macrocolonies was estimated by determining the phage titer of the supernatant of resuspended macrocolonies. Macrocolonies were resuspended in 1 ml TSB + 5 mM $CaCl_2$ at the indicated times. Serial dilutions of the solution were plated on TSB to calculate the CFU. To determine the phage titer, the cells were removed by centrifugation at 10,000 x g for 5 min. The supernatant was filtered and serial dilutions were made. The samples were used to transduce the strain RN4220 as described in the section IV.4.8. The plaque forming units (pfu) were counted at an appropriate dilution. The relative phage release was calculated by the ratio of pfu / CFU for each time point.

IV.3.11 H_2O_2 exposure

Cultures of USA300 were grown in TSB for 4 h to mid-exponential growth phase. H_2O_2 was added to final concentrations of 0.1% and 1% from a 30% stock. Cultures were grown with H_2O_2 for 2 h. The cells were harvested by centrifugation, resuspended in 30 µl TSB and 2 µl were spotted on TSBMg plates for macrocolony formation. The macrocolony morphology of H_2O_2-exposed cultures was compared to H_2O-exposed cultures after 5 days.

IV.3.12 Sucrose gradient

To analyze which components in the supernatant are responsible for lysis in the competing strain, it was prepared as described in section IV.3.9 and subjected to a sucrose gradient (Brakke, 1953, Hirst and Cox, 1976). Two milliliters of the supernatant were loaded onto the continuous 5% - 40% (w/v) sucrose gradient prepared in MES buffer. The tubes were centrifuged in a swing-out rotor at 100,000 x g at 4°C for 16 h. Ten fractions à 1.3 ml volume were aspired from the top and 5 µl of each were dripped onto macrocolonies 8 h after spotting. The occurrence of lysis was assessed after 3 days.

IV.4 Generation of genetically modified strains

IV.4.1 *E. coli* electrocompetent cells

The plasmid-free *E. coli* strain DH5α was used for cloning. For maximum transformation efficiency, electrocompetent cells were aliquoted and frozen in exponential growth phase. A preculture was grown overnight at 37°C and 10 ml were used to inoculate 1 l LB medium. The culture was grown at 37°C to an OD_{600} = 0.5 and growth was stopped by placing the culture on ice for 15-30 min. The cells were pelleted at 4000 x g for 15 min at 4°C. This was repeated after washing the cells with cold sterile dH_2O. The cells were resuspended in 10 ml dH_2O + 10% (v/v) glycerol and stored in 40 µl aliquots at -80°C.

IV.4.2 *E. coli* transformation

Six µl of a desalted ligation reaction (see IV.5.8) was added to an aliquot of cells thawed on ice. The mixture was added to a pre-cooled electroporation cuvette (Bio-Rad, 1 mm gap width) and electroporated with the voltage set to 1.8 kV and pulse duration 5 ms. Afterwards, 500 µl LB medium was added and cells were incubated at 37°C for 1 h. The cells were plated on LB with a selecting antibiotic and X-gal for color selection and incubated at 37°C for 1 day.

IV.4.3 *S. aureus* RN4220 electrocompetent cells

The *S. aureus* strain RN4220 is frequently used for transformation of non-staphylococcal DNA due to a defect in the restriction modification system. The efficiency of plasmid DNA uptake through electroporation can be further improved by a sucrose treatment of cells in early exponential growth phase.

An overnight preculture was used to start a culture with 100 ml volume at an OD_{600} = 0.05. The culture is incubated to early exponential growth phase with an optical density at 600 nm = 0.2-0.25 and harvested by centrifugation at 4500 x g for 10 min at 4°C. Cold 0.5 M sucrose solution was used for consecutive washing steps with 100 ml, 25 ml, 10 ml and 5 ml volume. Finally, the cells were resuspended in 1 ml of the solution and stored in 60 µl aliquots at -80°C.

IV.4.4 *S. aureus* transformation

Plasmid DNA for the transformation of RN4220 was isolated from *E. coli* DH5α using the NucleoSpin Plasmid MiniPrep kit (Macherey-Nagel) according to the manufacturer's instructions. The cells were thawed on ice and incubated with 1.5 µg plasmid DNA for 10 min. The mixture was transformed by electroporation with the same cuvettes and settings as described in the section IV.4.2 500 µl TSB was added to the cells and they were incubated at 30°C for 1 h. The cells were plated on TSB with erythromycin and X-gal to select plasmid-containing clones after 48 h at 30°C.

IV.4.5 *S. aureus* gene deletions

Deletions of the biofilm regulators in the strain USA300_TCH1516 were created using the pMAD vector system with a temperature-sensitive double recombination process (Arnaud et al., 2004). A cassette giving resistance to tetracycline was cloned into the multiple cloning site (MCS) using the primers CW114 + 115 and the restriction enzymes SalI-HF and EcoRI-HF yielding the vector pMADtet. 500 bp flanking regions of the target genes were amplified using the following primers: *pckA* (upstream: CW37+CW38 / downstream: CW39+CW40), *purK* (CW29+CW30 / CW31+CW32) and *srrA* (CW33+CW34 / CW35+CW36). The flanking regions were inserted up- and downstream of the *tet* cassette using BamHI-HF + SalI-HF and EcoRI-HF + BglII, respectively.

The plasmid was transformed into *E. coli* and *S. aureus* as described in the sections IV.4.2 and IV.4.4. To force integration of the plasmid into the genome, the clones were grown in 1 ml TSB at 42°C for 6 h. At this temperature, the pMAD plasmid does not replicate, thus only clones where integration of the plasmid into the genome has occurred maintain the antibiotic resistance. Serial dilutions were plated onto TSB + erythromycin + X-gal. After 48 h, light blue colonies carrying the pMAD plasmid in their genome were picked and used for phage transduction or the 2nd recombination step.

In the 2nd recombination step, the homologous regions on the plasmid and the genome recombine with two possible outcomes: The plasmid can be completely excised leaving

behind the WT situation or the targeted regions between the homologous sequences on the plasmid are excised and replaced by the antibiotic marker. For the 2nd recombination, a light blue colony from the 1st recombination was picked and grown at 30°C for 6 h. The culture was then shifted to 42°C for 3 h. Serial dilutions were plated on TSB + X-Gal and incubated at 42°C for 48 h. White colonies were streaked on TSB + erythromycin and TSB + tetracycline. In these colonies, the 2nd recombination occurred and they should hence be sensitive to erythromycin. Only in tetracycline-resistant clones, the 2nd recombination between the correct regions occurred and the target gene was replaced by the tetracycline-resistance cassette.

To make the double mutant *ΔsrrA Δagr*, *srrA* was also deleted with a spectinomycin resistance marker. The vector pMADspc was created analogous to pMADtet with the primers CW116 + 117 to clone the spectinomycin resistance cassette into the MCS of pMAD. All following steps performed as described for pMADtet using spectinomycin as the selecting antibiotic for the 2nd recombination.

IV.4.6 Complementation

To complement deletion strains, an enhanced version of the pMAD vector was used. The vector pAmy carries sequences that target it to the gene SA2244 of the *S. aureus* reference strain N315, which is annotated as a glutamyl-peptidase (Yepes et al., 2014). It was shown that insertion into the gene does not affect the fitness or phenotype. Thus, the locus is suitable for the stable integration of a plasmid into the genome. The genes to be complemented were expressed from their native promoters. For *pckA* and *srrA*, that are monocistronic or the first gene of the operon, the amplified fragments comprise approximately 500 bp upstream of the start codon and 100 bp downstream of the stop codon. The primers for *pckA* were CW138+139, for *srrA* CW140+141 and both fragments were cloned using the restriction enzymes BamHI-HF and SalI-HF.

For *purK*, a joining PCR between the promoter of the operon P_{purE} and *purK* was made (Wach, 1996). The reaction products to be joint in the PCR were amplified using the primers

CW146+144 (P_{purE}) and CW143+145 (purK). The joining of the fragments was achieved using the primers CW142+145 and the product was cloned into pAmy using the restriction enzymes NcoI-HF and SalI-HF.

The plasmids pAmy-P_{pckA}-pckA, pAmy-P_{purE}-purK and pAmy-P_{srra}-srrA were transformed into RN4220 and the first recombination was performed as described for the section IV.4.5. The constructs were shuttled to the respective deletion strains in USA300_TCH1516 using φ11 phages as described in the section IV.4.8. The second recombination of the complementation construct was performed as described in section IV.4.5.

IV.4.7 Heterologous expression of Stk2

For expression of *stk2* in the strain RN4220, the above-mentioned pAmy vector system was used (Yepes et al., 2014). The gene with its endogenous promoter was amplified from the genome of the strain N315 using the primers CW162 + 163. The fragment P_{stk2}-stk2 was cloned into the vector pAmy with the enzymes BamHI-HF + SalI-HF. Transformation of the plasmid into *S. aureus* and integration of the plasmid into the chromosome was performed as described earlier. The functionality of Stk2 was confirmed by the experimental finding that the strain RN4220 *amy::pAmyP$_{stk2}$-stk2* was resistant to the production of a phage lysate (Depardieu et al., 2016).

IV.4.8 Phage transductions

Constructs were shuttled from RN4220 to USA300_TCH1516 using transduction with the phage φ11 (Iandolo et al., 2002). Clones were selected using erythromycin (plasmid or 1st recombination) or tetracycline / spectinomycin (2nd recombination). The phage lysate was generated from cultures of donor strains in stationary phase. The culture was supplemented with 5 mM $CaCl_2$ and 300 µl of the culture was mixed with 100 µl of serial dilutions of the phage stock in a culture tube. The mixture was incubated at room temperature for 15 min. Liquid warm soft agar (0.5% (w/v) agar) was poured into the tube, inverted to mix and poured onto a TSB plate. The plates were incubated ON to allow for the appearance of lytic phage plaques in the lawn of cells. The plaques are caused by phages that infect the bacterial host

cells and enter the lytic cycle. The massive replication of the phages causes the bacteria to burst open and the released phages can infect neighboring cells. The phages were harvested from the plate with the highest dilution of the phage stock that still showed confluent lysis. To harvest the phages, 3 ml TSB + 5 mM CaCl2 were added onto the plate and the soft agar was scraped into a falcon tube. The sample was vortexed for 30 sec. To separate the agar and cellular debris from the phages, the sample was centrifuged at 4000 rpm for 15 min. The supernatant was sterilized by filtration (0.2 µm) and 3 µl chloroform were added to the bottom of the tube for conservation. The phage stocks can be stored at 4°C for several months.

The recipient strains for the phage transduction were grown in 2 ml TSB for 6 h. $CaCl_2$ was added to the culture to a final concentration of 5 mM. The recipient strains were heated to 57°C for 90 sec to improve uptake of foreign DNA and split into 300 µl aliquots. The cultures were incubated with 100 µl of the phage stock at different dilutions (10^0 to 10^{-2}) at room temperature for 15 min. Samples were plated on selection plates with 10 mM citrate to kill residual phages and incubated at 37°C for 48-96 h. Colonies were checked by colony PCR for successful transduction of the constructs.

IV.5 Molecular biology techniques

IV.5.1 Polymerase Chain Reaction (taq/phu)

The polymerase chain reaction (PCR) was used to amplify DNA fragments for cloning of vectors (Mullis and Faloona, 1987). The polymerases Taq or Phusion (New England BioLabs) were used to amplify from genomic DNA or plasmid DNA as a template. The primers used in this study are found in VII.3.

For Taq polymerase, the reaction was prepared as follows:

5 µl	10x Taq buffer
1 µl	DMSO
1 µl	10 mM dNTPs (dATP, dGTP, dTTP, dCTP)

0.5 µl	forward primer (10 µM)
0.5 µl	reverse primer (10 µM)
1 µl	template DNA
0.5 µl	Taq Polymerase
ad 50µl	dH_2O

The following cycling program was used for reactions with the Taq polymerase:

Initial denaturation	98°C	5:00	
Denaturation	98°C	0:45	35x
Annealing	54°C	0:45	
Extension	72°C	1:00 per 1 kb amplicon	
Final extension	72°C	7:00	

The Phusion polymerase possesses 3'-5' exonuclease activity for proofreading of the construct and higher speed so that reactions can be carried out faster. Reactions with the Phusion polymerase were prepared as follows:

10 µl	5x Phusion-HF buffer
1 µl	DMSO
1 µl	10 mM dNTPs (dATP, dGTP, dTTP, dCTP)
0.5 µl	forward primer (10 µM)
0.5 µl	reverse primer (10 µM)
1 µl	template DNA
0.5 µl	Phusion Polymerase
ad 50µl	dH_2O

The following cycling program was used for reactions with the Phusion polymerase:

Initial denaturation	98°C	5:00	
Denaturation	98°C	0:20	35x
Annealing	54°C	0:30	
Extension	72°C	0:20 per 1 kb amplicon	

Final extension	72°C	7:00

IV.5.2 Colony PCR

Short fragments up to approximately 1 kb can reliably be amplified directly from bacterial material in a colony PCR. Colonies were picked and resuspended in 15 µl H_2O. To break the cells, they were heated to 95°C for 5 min. One µl of the lysate was used as a template for conventional PCRs using Phusion or Taq polymerase as described in the section IV.5.1.

IV.5.3 Joining PCR

Long flanking homology PCR (LFH PCR) was developed to join two or more overlapping DNA fragments into a longer product (Wach, 1996). The fragments were amplified with an extension at the 3'-end that is homologous to the 5'-end of the fragment that they will be joint with. The reaction uses a special mix of several polymerases (Expand^{TM} long template PCR System, Sigma-Aldrich) and was prepared as follows:

250 µg	each DNA fragment as template
5 µl	10x Expand Long Template Buffer 2
2 µl	10 mM dNTPs (dATP, dGTP, dTTP, dCTP)
0.5 µl	forward primer (10 µM)
0.5 µl	reverse primer (10 µM)
0.5 µl	Expand Long Template Enzyme Mix
ad 50 µl	dH_2O

The LFH PCR uses a two-step cycling program with shorter extension times at the early cycles.

Initial denaturation	94°C	2:00	
Denaturation	94°C	0:10	10x
Annealing	54°C	0:30	
Extension	68°C	2:00 per 1 kb amplicon	

Denaturation	94°C	0:10	25x
Annealing	54°C	0:30	
Extension	68°C	2:20 per 1 kb amplicon	
Final extension	68°C	7:00	

IV.5.4 Plasmid isolation

Plasmid DNA was isolated from *E. coli* using the NucleoSpin Plasmid MiniPrep kit (Macherey-Nagel) according to the manufacturer's instructions for low copy plasmids. The cellular material was collected from agar plates that were incubated overnight at 37°C.

IV.5.5 Genomic DNA isolation

To isolate genomic DNA from *S. aureus*, cells were collected from an agar plate and resuspended in 700 µl lysis buffer. To break down the cell wall, 17.5 µl lysostaphyin (stock solution 1 mg/ml, Ambi Products) was added and incubated at 37°C for 15 min. RNA and proteins were degraded by the addition of RNaseA (10 µl of a 1 mg/ml stock solution, 10 min at 37°C) and proteinase K (10 µl of a 10 mg/ml stock solution, 15 min at 55°C). The cell lysate was mixed with equal volume phenol and vortexed well to mix the phases. To separate the phases, the solution was centrifuged at 21,100 x g for 5 min. The upper aqueous DNA-containing phase was transferred to a new tube and 70 µl sodium acetate (3 M) and 490 µl isopropanol were added to precipitate the DNA. It was pelleted by centrifugation with 21,100 x g for 15 min. The pellet was washed with 300 µl 70% (v/v) ethanol and centrifuged for another 10 min at 21,100 x g. The DNA pellet was air-dried and resuspended in 50 µl 50 mM Tris (pH 7.0).

IV.5.6 Isolation from agarose gel and PCR

To clean up DNA from agarose gels and PCR reactions, the NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel) was used according to the manufacturer's instructions. The process of DNA electrophoresis in TAE-based agarose gels is detailed in section IV.5.7.

IV.5.7 Agarose gel electrophoresis

The negative charge of DNA allows for a size-separation when exposed to an electric current. Electrophoresis was performed in TAE-based agarose gels (0.8% w/v). DNA samples were mixed with 3x DNA loading buffer. Five µl 1 kb DNA ladder was used as a standard. The samples were run with a constant voltage of 100 V for 30-45 min. The samples were imaged using the Gel iX Imager system (Intas Science Imaging).

IV.5.8 Restriction digestion and ligation

For cloning the vectors, the plasmids and inserts were digested with restriction enzymes and afterwards ligated. The restriction enzymes BamHI-HF, BglII, EcoRI-HF, NcoI-HF and SalI-HF as well as the T4 DNA ligase were purchased from New England BioLabs (NEB). The restriction digestion reaction was prepared as follows:

1 µg	DNA
5 µl	10x supplied reaction buffer
1 µl	each restriction enzyme in the reaction
ad 50 µl	dH_2O

The reaction was incubated at 37°C for 1 h. The digested contents were purified using the NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel) according to the manufacturer's instructions. If the restriction enzymes could not be used simultaneously due to incompatibility of the reaction buffer, the reaction was performed with one restriction enzyme and repeated with the other enzyme after purification with the clean-up kit.

To join the digested inserts and plasmids by forming a phosphodiester bond between the matching cohesive ends, a ligation reaction was performed at 18°C overnight. The reaction partners were mixed at a molar insert:vector ratio of 1:1, 3:1 or 5:1. The reaction volume was 20 µl with 2 µl of the supplied 10x reaction buffer and 0.8 µl T4 DNA Ligase. To improve transformation efficiency, the reaction was desalted against water on nitrocellulose membranes for 10 min.

IV.5.9 Sequencing

To verify correct construction of plasmids, their sequence was confirmed by Sanger sequencing service by GATC Biotech (Konstanz) (Sanger et al., 1977). Five µl plasmid DNA of which the concentration was adjusted to 80-100 ng/µl was mixed with 5 µl primers (5 µM) and sent for sequencing. This method typically gives reads with a maximum of 1000 nt. Therefore, several primers were used to achieve complete coverage of sequences with more than 1000 nt.

IV.5.10 Mapping of transposon insertion sites

The correct insertion of the *bursa aurealis* transposon in the annotated genes was confirmed for the candidates identified in the NTML screen. Information on the orientation of the genes and transposon was retrieved from http://app1.unmc.edu/fgx/ (Fey et al., 2013). Gene specific primers were designed to bind 150 – 400 bp from the annotated transposon insertion site. The Tn-specific primers Tn Upstream and Tn Buster for genes with the transponson in "plus" and "minus" orientation, respectively, were used as suggested by Fey, et al. 2013. The Tn/chromosome junctions were PCR-amplified using transposon- and gene-specific primers and sequenced. The sequences were aligned to the respective genes to confirm the transposon insertion sites. All primers and the predicted PCR-fragment sizes are listed in section VII.3.

IV.6 Biochemistry

IV.6.1 Extracellular matrix extraction

The extraction of the extracellular matrix (ECM) from the macrocolonies was based on a previously published protocol by Chiba et al (2015). The method uses high concentrations of NaCl to break the bonds between bacteria and the non-cellular material of the extracellular matrix. Macrocolonies were grown for five days before harvesting the material to extract the matrix. Three macrocolonies were scraped off the plates into 1 ml PBS and collected by centrifugation at 10.000 x g for 5 min. Cells were resuspended in 750 µl 1.5 M NaCl and briefly (10 s) sonicated at the lowest amplitude to disperse the aggregate (Sonopuls,

Bandelin; probe: MS73). The OD_{600} was measured and normalized to 1 ml of OD_{600} = 10 with 1.5 M NaCl. Cells were collected by centrifugation as above and the supernatant was recovered as the isolated ECM. The concentration of proteins and eDNA in the fraction was determined by spectrophotometer (Nanodrop™ 1000, Thermo Fisher Scientific). The concentration of saccharides in the ECM fraction was determined as described unterhalb.

IV.6.2 Saccharide quantification

Saccharides in the extracted extracellular matrix were quantified colometrically using the phenol-sulfuric acid method (Nielsen, 2010). Briefly, 143 µl of the matrix fraction was mixed with equal amounts of 5% (v/v) phenol. The volume of the sample was adjusted to 1 ml with 714 µl sulfuric acid. The tubes were inverted to mix the components and then incubated for 10 min at 25° C. Absorption at 492 nm was measured to determine the saccharide content of the sample. Glucose in the concentrations 0.05 – 2 mg/ml was used as a standard to create a calibration curve.

IV.6.3 Protein precipitation

To increase the protein concentration in solutions, the proteins were precipitated with trichloroacetic acid. Trichloroacetic acid from a 100% (w/v) solution was added to the protein solution to a final concentration of 5% and the samples were stored at 4°C overnight. The proteins were pelleted by centrifugation at 20,000 x g at 4°C for 15 min. To remove residual precipitant, the samples were washed twice with 500 µl cold acetone and centrifuged as described above. The proteins were resuspended in 20 µl 1x Laemmli buffer and denatured at 95°C for 5 min before SDS-Page (Laemmli, 1970).

IV.6.4 SDS-PAGE

Proteins were separated according to size by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using equipment by Bio-Rad. The proteins were focused in a stacking gel (6.7% acrylamide) that was prepared as follows:

2.5 ml dH_2O

1.5 ml 0.5 M Tris-HCl, pH 6.8

0.6 ml acrylamide/bis 37.5:1, 30%

50 µl 10% (w/v) SDS

20 µl 10% (w/v) APS

20 µl TEMED

Separation of proteins was performed in a 10% acrylamide gel that was prepared as follows:

4.9 ml dH_2O

2.5 ml 0.5 M Tris-HCl, pH 6.8

2.5 ml acrylamide/bis 37.5:1, 30%

100 µl 10% (w/v) SDS

50 µl 10% (w/v) APS

20 µl TEMED

Ten µl of the samples was loaded per well and samples were run into the gel with constant voltage of 100 V for 10 min. To separate the proteins, constant voltage of 150 V was used. When required a broad range prestained protein marker (ColorPlus, NEB) was used to estimate the size of the proteins.

IV.6.5 Mass Spectrometry

For mass spectrometry, a core facility service at the University of Würzburg was used. The samples were run on separating SDS-gels for approximately 1 cm. The gel was stained with fresh Coomassie staining solution for 30 min and afterwards destained with Coomassie destaining solution for 2 h. The sample was excised from the gel in 3 horizontal stripes of similar size using a sterile scalpel.

IV.7 Image acquisition

Images of macrocolony aggregates were captured under a SMZ1500 stereomicroscope (Nikon) with an Axiocam Color 412-312 (Zeiss) at the lowest magnification. The utilized software was AxioVision 4.7.2 (Zeiss).

Images of lysed macrocolonies in the supernatant assay were captured using a digital camera in front of a black background for improved contrast of the colonies.

IV.8 Gene ontology analysis

The candidates identified in the screen of the NTML were grouped according to gene ontology (GO) terms. The information was collected in the UniProt database (www.uniprot.org). Whenever possible, reviewed database entries of USA300 isolates were used. The strain COL was used as an alternative if the entry for USA300 was not reviewed. As the information regarding the cellular compartment was not available for all candidates, we additionally predicted intra- and transmembrane domains. Proteins without such predicted domains were classified as cytoplasmatic. The information regarding the biological processes was extracted from the same database.

IV.9 *Galleria mellonella* infection model

The larvae of the wax moth *Galleria mellonella* were infected with USA300_TCH1516 WT and the novel biofilm modulators to assess if virulence is affected in the candidates. *G. mellonella* larvae in the last larval developmental stage were purchased from Live Bait (Ex Balk, Netherlands). USA300 WT and the knockout variants were grown in 2 ml brain-heart-infusion medium (BHI) overnight. The OD_{600} was measured and precultures were started with an OD_{600} = 0.05 in 20 ml BHI. The cultures were incubated at 37°C for 1.5-2 h until they reached an OD_{600} = 0.6-0.8. Cells were pelleted at 4°C at 4.000 x g for 10 min and washed twice with 10 mM $MgSO_4$. Finally, the OD_{600} was adjusted to 0.3. Cohorts of 15 larvae were injected with 20 µl cell suspension in the last proleg using an insulin pen (BD Micro-Fine 0.3 ml for U100 insuline, Becton Dickinson). The infection dosage corresponds to 1.5 x 10^6 CFU. The control group was injected with 20 µl 10 mM $MgSO_4$. The larvae were incubated at 37°C for 48 h and survival was monitored after 24 h and 48 h. Larvae were scored “dead” when they turned black and did not respond to tapping anymore.

IV.10 Statistical analysis

All statistical analyses were performed using the software Prism (GraphPad). Replicates were performed as stated in the legend of the respective figures. Graphs represent the mean ± standard deviation. The unpaired Student's t-test was performed for statistical comparison of two groups. Differences were considered significant for a p-value ≤ 0.05.

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VI. Abbreviations

% (v/v)	% (volume/volume)
% (w/v)	% (weight/volume)
(p)ppGpp	guanosine tetraphosphate/pentaphosphate
AIR	5-aminoimidizole ribonucleotide
AMP	adenosine monophosphate
APS	ammonium persulfate
ATP	adenosine triphosphate
BHI	brain-heart infusion
bp	base pair
CAIR	5'-phosphoribosyl-4-carboxy-5-aminoimidazole
c-di-AMP	cyclic di-adenosine monophosphate
CDS	coding DNA sequence
CFU	colony forming units
cm	chloramphenicol
CV	crystal violet
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
dTTP	deoxythymidine triphosphate
ECM	extracellular matrix
eDNA	extracellular DNA
g	relative centrifugal force
gDNA	genomic DNA
GO	gene ontology
GTP	guanosine triphosphate
HK	histidine kinase
ica	intercellular adhesin
IMP	inosine monophosphate
kb	kilo base
LB	lysogeny broth

LFH-PCR	long flanking homology polymerase chain reaction
M	molar
mls	macrolide lincosamide streptogramin
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
MSSA	methicillin-sensitive *Staphylococcus aureus*
NCAIR	N5-carboxy-aminoimidazole ribonucleotide
nt	nucleotide
NTML	Nebraska transposon mutant library
OD	optical density
ON	overnight
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
pfu	plaque forming units
PNAG	poly-β(1,6)-N-acetyl-D-glucosamine
PRPP	5-phosphoribosyl-pyrophosphate
RNase	ribonuclease
RR	response regulator
SCV	small colony variant
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
spc	spectinomycin
TA	teichoic acids
TCA	tricarboxylic acid
TCS	two-component system
TEMED	Tetramethylethylenediamine
tet	tetracycline
Tn	transposon
Tris	tris(hydroxymethyl)aminomethane
TSB	tryptic soy broth
WT	wild type
WTA	wall teichoic acids
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

VII. Appendix A

VII.1 List of strains

Organism	Genotype	Strain #	Reference
Wild types			
E. coli DH5α	wild type	CW289	(Reusch et al., 1986)
S. aureus RN4220	wild type	CW106	(Kreiswirth et al., 1983)
S. aureus Newman	wild type	CW60	(Duthie and Lorenz, 1952)
S. aureus USA300_TCH1516	wild type	CW108	(Gonzalez et al., 2005)
S. aureus USA300-JE2	wild type	CW58	(Fey et al., 2013)
S. aureus RN450	wild type	CW295	(Novick, 1967)
Transposon mutants			
S. aureus USA300-JE2	*ΩqoxA::tn*	NE92	(Fey et al., 2013)
	ΩfakA::tn	NE229	
	ΩtcaA::tn	NE285	
	ΩhemL::tn	NE303	
	ΩfumC::tn	NE427	
	ΩpurB::tn	NE522	
	ΩSAUSA300_1969::tn	NE523	
	ΩpurA::tn	NE529	
	Ωfbp::tn	NE542	
	ΩribBA::tn	NE573	
	ΩpurF::tn	NE581	
	ΩgltA::tn	NE594	
	ΩpurK::tn	NE744	

Organism	Genotype	Strain #	Reference
	ΩctaA::tn	NE769	
	ΩrecA::tn	NE805	
	ΩclpP::tn	NE912	
	ΩpurC::tn	NE950	
	Ωmqo::tn	NE1003	
	ΩSAUSA300_0538::tn	NE1071	
	ΩpurS::tn	NE1134	
	ΩpckA::tn	NE1260	
	ΩsrrA::tn	NE1309	
	ΩribH::tn	NE1318	
	ΩodhB::tn	NE1391	
	ΩctaB::tn	NE1434	
	ΩcrtM::tn	NE1444	
	ΩpurL::tn	NE1464	
	ΩSAUSA300_1008::tn	NE1465	
	ΩlpdA::tn	NE1610	
	ΩSAUSA300_1118::tn	NE1735	
	ΩmpfA::tn	NE1847	
Genetically modified *S. aureus* strains			
S. aureus Newman	*ΔsigB::mls*	CW105	(Koch et al., 2014b)
S. aureus Newman	*Δagr::tet*	JC149	(Garcia-Betancur et
S. aureus Newman	*Δspa::spc Δica::cm*	JC301	al., 2017)
S. aureus USA300_TCH1516	*ΔpckA::tet*	CW134	This work
S. aureus USA300_TCH1516	*ΔpurK::tet*	CW151	This work
S. aureus USA300_TCH1516	*ΔsrrA::tet*	CW153	This work
S. aureus USA300_TCH1516	*ΔsigB::mls*	CW131	This work
S. aureus USA300_TCH1516	*ΔpckA::tet*	CW480	This work

Organism	Genotype	Strain #	Reference
	amy::P_{pckA}-pckA		
S. aureus USA300_TCH1516	*ΔpurK::tet*	CW485	This work
	amy::P_{purE}-purK		
S. aureus USA300_TCH1516	*ΔsrrA::tet*	CW482	This work
	amy::P_{srrA}-srrA		
S. aureus USA300_TCH1516	*Δagr::tet ΔsrrA::spc*	CW471	This work
S. aureus USA300_TCH1516	*Δagr::tet*	CW422	This work
S. aureus USA300_TCH1516	*ΔpckA::tet ΔsigB::mls*	CW464	This work
S. aureus USA300_TCH1516	*ΔpurK::tet ΔsigB::mls*	CW477	This work
S. aureus USA300_TCH1516	*ΔsrrA::tet ΔsigB::mls*	CW466	This work
S. aureus RN450	φ11	CW296	(Maiques et al., 2006)
S. aureus RN450	φ80α	CW297	(Selva et al., 2009)
S. aureus RN450	φ85	CW298	
S. aureus RN4220	*amy::pAmy-P_{stk2}-stk2*	CW445	This work

VII.2 List of plasmids

Name	Description	Reference
pMAD	temperature sensitive plasmid system to create deletions in *S. aureus*	(Arnaud et al., 2004)
pMADtet	pMAD derivative with a tetracycline resistance gene in the MCS	This work
pMADspc	pMAD derivative with a specinomycin resistance gene in the MCS	This work
pMADΔ*pckA::tet*	pMAD derivative for deletion of *pckA* with tetracycline resistance marker	This work
pMADΔ*purK::tet*	pMAD derivative for deletion of *purK* with tetracycline resistance marker	This work
pMADΔ*srrA::tet*	pMAD derivative for deletion of *srrA* with tetracycline resistance marker	This work
pMADΔ*srrA::spc*	pMAD derivative for deletion of *srrA* with spectinomycin resistance marker	This work
pAmy	pMAD derivative that integrates in the amylase gene of *S. aureus* (SA2244)	(Yepes et al., 2014)
pAmyP_{pckA}-*pckA*	pAmy derivative harboring promoter region and gene *pckA*	This work
pAmyP_{purE}-*purK*	pAmy derivative harboring promoter region of *pur* operon and gene *purK*	This work
pAmyP_{srrA}-*srrA*	pAmy derivative harboring promoter region of *srrAB* and gene *srrA*	This work
pAmyP_{stk2}-*stk2*	pAmy derivative harboring promoter region and gene *stk2*	This work

VII.3 List of primers

Primers for cloning

Function	Name	Sequence (5‘ – 3‘)
upstream flanking region of *pckA*	CW37	AAAAAGGATCCAAGCTACACGTGCATTTGG
	CW38	TTTTTGTCGACCCTAAATCCCTCCAAAGCG
downstream flanking region of *pckA*	CW39	AAAAAGAATTCATTTGAATACTAAATCAAAACC
	CW40	TTTTAGATCTTATTGCGTCAATTATGTGAAAG
upstream flanking region of *purK*	CW29	AAAAAGGATCCCACAATTCAAACTTTTGAAAGG
	CW30	AAAAAGTCGACTTTGCATGTCCTCCACTTTTTG
downstream flanking region of *purK*	CW31	AAAAAGAATTCAGCATGACATTATTATATG
	CW32	TTTTAGATCTATAGCATCCATTAATTGTTTC
upstream flanking region of *srrA*	CW33	AAAAAGGATCCTAATGACACATCCAAGATATC
	CW34	TTTTTGTCGACACAGGTCATACCTCCCACAC
downstream flanking region of *srrA*	CW35	AAAAAGAATTCTGTCGTAATTAAACTGTGG
	CW36	TTTTAGATCTTAATAGCATTATTTGTATCTTC
promoter region and gene *pckA*	CW138	AAAAAGGATCCCTTTCGGAATATCAACATATGTTG
	CW139	AAAAAGTCGACGAATATTTAGATCCTTTCACTGG
promoter region and gene *srrA*	CW140	AAAAAGGATCCGCACGTATTTATCCTGTTGGTCG
	CW141	AAAAAGTCGACGCTTCTTCTTTATTATGTGATTG
promoter region P_{purE} for LFH-PCR	CW146	AAAAACCATGGCGCTAACTTGTTTTGGTAATGG
	CW144	TATTGAAGTTCATTTTGCCAACTCTCTTGCATAATT
gene *purK* for LFH-PCR	CW143	AATTATGCAAGAGAGTTGGCAAAATGAACTTCAATA
	CW145	AAAAAGTCGACGATTATTTAATCGCCCCTTACCTG
tetracycline cassette into MCS of pMAD	CW114	AAAAGTCGACTCTTGCAATGGTGCAGGTTGTTC
	CW115	TTTTGAATTCGAACTCTCTCCCAAAGTTGATCC
spectinomycin cassette into MCS of pMAD	CW116	AAAAGTCGACGACTGGCTCGCTAATAACGTAACG
	CW117	TTTTGAATTCCGTAGCGAGGGCAAGGGTTTATTG
promoter region and gene *stk2*	CW162	AAAAGGATCCCTACAGTTTTGTACTTATGCTCAG
	CW163	TTTTGTCGACCAAATTATTATCTCCTCATAC

Primers for sequencing of transposon insertion

Gene [fragment size]	Name	Sequence (5' – 3')
Tn Upstream [adds 464 bp]	CW45	CTCGATTCTATTAACAAGGG
Tn Buster [adds 133 bp]	CW46	GCTTTTTCTAAATGTTTTTTAAGTAAATCAAGTAC
Tn Seq purA [533 bp]	CW168	TTGATGTAACGAATGGATATGTACCATG
Tn Seq SAUSA300_0538 [864 bp]	CW169	TTTATCTATGAAGCTTGTATAATGACCC
Tn Seq mpfA [863 bp]	CW170	TTCATAGTTTAATATCACCCAAATAATTTG
Tn Seq clpP [867 bp]	CW171	TTTCAATACTTTGACCAGTACGCTCTG
Tn Seq qoxA [497 bp]	CW172	AAGTGTCAAAATTTAAGTCTTTGCTTCTAT
Tn Seq purK [867 bp]	CW173	TTTTATCTATTCTCGCTGGAACAATTG
Tn Seq purC [533 bp]	CW174	TTCAATATCTTCATCTGATGCTATATTG
Tn Seq purS [709 bp]	CW175	TTATGCATTCTCCTTTTCATCATCTAA
Tn Seq purL [535 bp]	CW176	ATAACGCGCTTCACCATCAATTGTTG
Tn Seq purF [871 bp]	CW177	TACCAGAAGCTTTACGTACTGCATG
Tn Seq lpdA [859 bp]	CW179	TACTGCTGGCATACCAATGTAATCAAC
Tn Seq SAUSA300_1008 [865 bp]	CW180	TGTATCCAAAATATTAATACGTGTACC
Tn Seq ctaA [521 bp]	CW181	AAAGCCGGAATTAAGTTACCTTGAAC
Tn Seq ctaB [866 bp]	CW182	ACGAAAAAGATCACTAAATAATTTAGTG
Tn Seq SAUSA300_1118 [628 bp]	CW183	TAAGCTTTCTTGCCTGTATTATTCACA
Tn Seq fakA [533 bp]	CW184	CATTAATGACTAATAAAGAATCACCAAATT
Tn Seq odhB [531 bp]	CW185	AAAGTTCCAGAATTAGCAGAATCTATTAC
Tn Seq srrA [618 bp]	CW186	ATGTCGAACGAAATACTTATCGTAGATG
Tn Seq hemL [748bp]	CW187	ATGAGATATACGAAATCAGAAGAAGCAATG
Tn Seq gltA [764 bp]	CW188	ATGGCAGAATTACAAAGAGGTTTAGAAG
Tn Seq ribH [533 bp]	CW189	TGGCATGATTATTTATCTACCTCAAGAAG
Tn Seq ribBA [534 bp]	CW190	ATTAGGAGATAAAGTACATTTAGAAACAG

Gene [fragment size]	Name	Sequence (5’ – 3’)
Tn Seq pckA [862 bp]	CW191	AAGATTAATTGCTTTTGCATAGCAGCC
Tn Seq fumC [864 bp]	CW192	ATTTGTTGTTTATTAATAACAGCAATATTG
Tn Seq purB [433 bp]	CW193	ATGATTGAACGCTATTCTAGAGAAGAAATG
Tn Seq tcaA [812 bp]	CW194	ATGAAATCTTGCCCGAAGTGCG
Tn Seq mqo [533 bp]	CW195	AAGGGGGACTGTATTTGTTATGACAAC
Tn Seq fbp [524 bp]	CW196	AAATTCGAAGTAAGTTTGCTAAGCATAC

VII.4 List of figures

VII.5. List of tables

VIII. Appendix B

VIII.1 Affidavit

I hereby confirm that my thesis entitled "*Morphology, regulation and interstrain interactions in a new macrocolony biofilm model of the human pathogen Staphylococcus aureus*" 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.

Würzburg, April 13, 2018 _____

Charlotte Wermser

Hiermit erkläre ich an Eides statt, die Dissertation „*Morphologie, Regulation und stammübergreifende Wechselwirkungen in einem neuen Makrokolonie-Biofilmmodell des Humanpathogens Staphylococcus aureus*" 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.

Würzburg, 13. April 2018 _____

Charlotte Wermser

VIII.2 Publications

Publications directly related to this work

Wermser C, Lopez D. Identification of *Staphylococcus aureus* genes involved in the formation of structured macrocolonies. Microbiology 2018 April 11. [Epub ahead of print] doi:10.1099/mic.0.000660

Publications unrelated to this work

Koch G, **Wermser C**, Acosta IC, Kricks L, Stengel ST, Yepes A, Lopez D. Attenuating *Staphylococcus aureus* Virulence by Targeting Flotillin Protein Scaffold Activity. Cell Chemical Biology 2017. Jul 20;24(7):845-857.e6.

Wermser C, Lopez D. Purification of Lipid Rafts from Bacterial Membranes. Hydrocarbon and Lipid Microbiology Protocols. Totowa, NJ: Humana Press; 2015:1-10.

Koch G, Yepes A, Forstner KU, **Wermser C**, Stengel ST, Modamio J, Ohlsen K, Forster KR, Lopez D. Evolution of Resistance to a Last-Resort Antibiotic in Staphylococcus aureus via Bacterial Competition. Cell 2014;158:1060-71

Active Participation in international symposia

Wermser C, Lopez D. "Genes Involved in the Formation of Multicellular Aggregates in *Staphylococcus aureus*" *VAAM 2015, March 1-4, 2015; Marburg/Germany* – Oral Presentation

Wermser C, Lopez D. "Genes Involved in the Formation of Multicellular Aggregates in *Staphylococcus aureus*" *4th Mol Micro Meeting 2015, September 16-18, 2015; Vienna/Austria* – Poster Presentation

Wermser C, Lopez D. "Formation and Integrity of Multicellular Aggregates in *Staphylococcus aureus*" *VAAM 2016, March 13-16, 2016; Jena/Germany* – Oral Presentation

Wermser C, Garcia-Betancur JC, Lopez D. "Cell-fate decision influences the infection lifestyle of *Staphylococcus aureus*" *Progress Report of the DFG Priority Program SPP 1617.April 19-21, 2016; Düsseldorf/Germany* – Oral Presentation

Wermser C, Lopez D. "Formation and Integrity of Multicellular Aggregates in *Staphylococcus aureus*" *Eureka! 11th International GSLS Student Symposium 2016, October 12-13, 2016; Würzburg/Germany* – Poster Presentation

Wermser C, Lopez D. "Genetic Control of the Formation of Multicellular Aggregates in *Staphylococcus aureus*" *5th Joint Conference of the DGHM&VAAM 2017, March 5-8, 2017; Würzburg/Germany* – Poster Presentation

Wermser C, Garcia-Betancur JC, Lopez D. "Cell-fate decision defines acute and chronic infection cell types in *Staphylococcus aureus" Progress Report of the DFG Priority Program SPP 1617. March 27-29, 2017; Hohenkammer/Germany* – Poster Presentation

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VIII.4 Curriculum Vitae