Analysis of the *Chlamydophila pneumoniae* and host transcriptome in the acute and iron depletion-mediated persistent infection

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The only constant

in the world is change.

To my parents,

and my friends.

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Selbständigkeitserklärung

Hiermit erkläre ich, daß ich die vorliegende Arbeit selbständig und nur mit den angegebenen Hilfsmitteln erstellt habe.

Ich erkläre außerdem, dass diese Dissertation weder in gleicher noch in anderer Form bereits in einem Prüfungsverfahren vorgelegen hat.

Ich habe früher, außer den mit dem Zulassungsgesuch urkundlichen Graden, keine weiteren akademischen Grade erworben oder zu erwerben gesucht.

André Mäurer

Berlin/Würzburg, den 29.04.2006

Zusammenfassung

Das obligat intrazelluläre, gram-negative Bakterium *Chlamydophila pneumoniae* (*Cpn*) wird mit akuten und chronischen Krankheiten in Verbindung gebracht. Besonders sein Potential persistente Infektionen zu durchlaufen ist mit chronischen Krankheiten korreliert worden und deshalb von besonderem Interesse. Verschiedene *in vitro* Zellkulturmodelle werden verwendet um persistente Infektionen zu untersuchen, darunter IFNγ Stimulation, Antibiotika Behandlung und Eisenmangel. Das letztere imitiert den körpereigenen Effekt, durch Limitierung des verfügbaren Eisens Infektionen zu bekämpfen. Über die Genregulation von *Cpn* als auch der Wirtzelle in der akuten und persistenten Infektion ist jedoch wenig bekannt. Deshalb wurde in dieser Arbeit das *Cpn* Transkriptom als auch das von epithelialen Wirtszellen untersucht. Zu diesem Zweck wurde ein Oligomikroarray, für die Stämme *CWL029*, *AR39* und *J138*, in Kooperation mit MWG AG entwickelt und validiert. Nur ein kleiner Teil der extrahierten GesamtRNA von *Cpn* infizierten Zellen stammt vom Pathogen. Ein optimierte RNA Extraktionsmethode führte zu der dreifachen Menge an bakterieller RNA in der GesamtRNA. Dies ermöglichte es, das *Cpn* Transkriptom ohne Amplifikation oder Anreicherung der Retikulärkörperchen (RK) zu untersuchen. Im Vergleich zu Studien für *C. trachomatis* (*Ctr*) stellt diese eine erhebliche methodische Verbesserung dar. Die Mikroarrayergebnisse wurden mittels Echtzeit-PCR (qRT-PCR) verifiziert, bei welcher die Genexpression mit Hilfe eines Indexes verschiedener Kontrollgene normalisiert wurde. Für die Mikroarraystudien wurde durch den Vergleich der Expressionswerte zu einem mittleren Zeitpunkt im Entwicklungszyklus die Expressionsprofile der Gene definiert. Mittels eines Algorithmuses für ‚selbstorganisierende Netzwerke' (SOM) wurden signifikant regulierte Gene aufgrund ihres Expressionsprofiles in 12 Cluster gegliedert. Während in der Literatur der Startpunkt der Expression für die Klassifizierung der Gene verwendet wurde, basiert dies hier auf dem Expressionsprofil der Gene. Diese 12 Cluster wurden wiederum in die Klassen der 'Frühen' (engl.: 'Early'), 'Mittleren' (engl.: 'Mid') und 'Späten' (engl.: 'Late') Gene eingeteilt. Diese Unterteilung lehnt sich an schon beschriebenen Genexpressionsstudien für *Ctr* an. Weiterhin wurde die neue Klasse der 'Verspäteten' (engl.: 'Tardy') Gene eingeführt. Diese hatten am Ende des Entwicklungszykluses ein kontinuierlich ansteigendes Expressionsprofil. Mit publizierten Proteinen aus chlamydialen Elementarkörperchen (EK) korrelierten vor allem Gene aus den 'Späte' jedoch nicht aus den 'Verspätete' Klassen. Gene dieser beiden Klassen müssen also eine unterschiedliche Rolle im EK Redifferenzierungsprozess spielen. Weiterhin waren überdurchschnittlich viele mRNA Transkripte aus der Klasse der 'Verspäteten' Gene in den EK vorhanden. Dies führte zu der Annahme, daß ein Teil der initiale Proteinexpression von stabilen mRNA-Transkripten aus der infektiösen EK Form erfolgt. Anschließend wurden, Gene, die für spezifische Signalwege und physiologische Funktionen von *Cpn* kodieren, basierend auf der ‚Gene Ontology' während des Entwicklungszykluses untersucht. Weiterhin wurde das Transkriptom von *Cpn* in der Persistenz mit dem Transkriptom der akuten Infektion

verglichen. Unter Persistenzbedingungen zeigte *Cpn* ein verändertes Expressionsprofil. Hochregulierte Gene konnten akuten Clustern am Anfang des akuten Entwicklungszykluses und herunterregulierte Gene Clustern am Ende des akuten Entwicklungszykluses zugeordnet werden konnten. Dies legt nahe, daß es sich bei der Persistenz nicht um ein neues Transkriptionsprofil handelt, sondern eher um eine Arretierung des Transkriptomes in der Mitte des akuten Entwicklungszykluses. Weiterhin zeigten konvergent und divergent orientierte Gene am Anfang des Zyklus bevorzugt ein antagonistisches Expressionsprofil, während in Reihe angeordnete ('tandem') Gene ein korreliertes Expressionsprofil aufwiesen. Bei den mit dem *Cpn* Stamm *CWL029* durchgeführten Mikroarrayexperimenten konnten auch Expressionswerte für einige ausschließlich für die Stämme *AR39* und *J138* beschriebenen Gene gemessen werden. Ein Vergleich mittels BLAST zeigte, daß diese Gene auch im *CWL029* Genom kodiert sind. Dazu gehörten mehrere Gene, welche konvergent zu ihren Nachbargenen orientiert waren und eine Sequenzüberlappung mit diesen aufwiesen. Darunter fielen *parB*, welches eine Rolle für die Trennung der DNA in der Zellteilung spielt, und *rpsD*, ein alternativer Sigma-Faktor, der für die Transkription in der späten Phase des Entwicklungszyklus verantwortlich ist. Für beide Genpaare konnte in der frühen akuten und in der persistenten Infektion ein antagonistisches Expressionsprofil beobachtet werden, wie es bei konvergent orientierten Genpaaren überwiegt. Mittels quantitativer qRT-PCR wurde für *rpsD* gezeigt, dass vollständige mRNA-Fragmente in der Persistenz herunterreguliert, während kurze mRNA-Fragmente hochreguliert waren. Als Erklärung für diesen Effekt dient ein Modell, welches auf einer Kollision der RNA Polymerasen basiert. Dieser Sigma-Faktor unabhängige Mechanismus ist in der Literatur als 'Transkriptionelle Interferenz' bekannt und führt so trotz einer Promoteraktivierung zu einer verminderten Anzahl an vollständigen mRNA Transkripten. Die Herunterregulation von RpsD auf Proteinebene in der *Cpn* Persistenz ist beschrieben worden. Im letzten Teil dieser Arbeit wurde das Wirtszelltranskriptom in der akuten und persistenten Infektion untersucht. Infektion mit *Cpn* führte zu einer Hochregulation von *relB*, welches an alternativen NF-κB Signalwegen beteiligt ist und das anti-apoptotische Potential verstärkt. Weiterhin waren Gene differentiell exprimiert, welche für die Zellzyklusproteine Cyclin-G2 und Cyclin-D1 sowie Inhibitoren von CDK4 kodieren. Sehr wahrscheinlich führt dies zu einem G1-S Übergang im Zellzyklus der Wirtszelle. Interessanterweise sind viele dieser Gene im persistenten Zustand stärker reguliert als in der akuten Infektion. Verschiedene Metabolittransporter waren differentiell reguliert, darunter *aquaporin-7*, welches nur in der Persistenz eine verminderte Expression zeigte. Die bei einer *Cpn* Infektion differentiell exprimierten Wirtsgene sind damit an anti-apoptotischen Mechanismen, am Zellzyklus, Zellproliferation und am Zellmetabolismus beteiligt. Zusammenfassen gibt diese Arbeit gibt einen Einblick sowohl in das Transkriptom des Pathogens als auch der Wirtszelle während der akuten und durch Eisenmangel ausgelösten persistenten Infektion als auch potentiellen Mechanismen zu Persistenzentstehung auf der Ebene der Genregulation.

Summary

The obligate intracellular gram-negative bacterium, *Chlamydophila pneumoniae* (*Cpn*), has a significant impact as an acute and chronic disease-causing pathogen. Its potential to undergo persistent infections has been linked to chronic diseases. Several *in vitro* cell culture models are used to study persistent conditions, mainly IFNγ stimulation, treatment with antibiotics and iron depletion. The latter mimics the body's potential to limit bacterial infections by iron withdrawal. Little is known about changes in the *Cpn* transcriptome during the acute and persistent infection. Therefore, the *Cpn* transcriptome during its acute developmental cycle and iron depletion-mediated persistence was examined in this study. To this end, an oligonucleotide microarray spanning the entire *Cpn* genomes of the strains *CWL029*, *AR39* and *J138* was designed. Only a marginal part of the total RNA is of bacterial origin. Therefore, the RNA extraction protocol was optimized and resulted in a 3 x fold increased yield for pathogen RNA. This allowed investigating the *Cpn* transcriptome without the use of RNA amplification or accumulation of the pathogen and represents a major improvement compared to earlier microarray studies done with *C. trachomatis* (*Ctr*). Microarray results were verified using real time PCR (qRT-PCR) in which gene expression was normalized to a housekeeping index (HKI) consisting of several reference genes. The expression of individual genes throughout the *Cpn CWL029* developmental cycle was compared to expression at a midpoint in the developmental cycle to generate expression profiles. Based on these profiles, genes with similar expression changes formed 12 clusters using the self-organizing map algorithm. While other studies define genes based on their onset of transcription, here the important feature for clustering was the expression profile. This turned out to be more appropriate for comparing the time specific relevance of a certain cluster of genes to their proposed functions in the cycle. The *Cpn* clusters were grouped into the 'Early', 'Mid' and 'Late' classes as described for *Ctr*. Additionally, a new gene expression class containing genes with steadily increasing expression at the end of the developmental cycle was defined and termed 'Tardy' class. Comparison of the *Cpn* clusters to published proteomics data showed that genes encoding elementary body (EB) proteins peaked in the 'Late' gene cluster. This indicated that genes of the 'Late' and 'Tardy' class have different roles in RB to EB re-differentiation. Moreover, using lexical comparison the EB mRNA profile was significantly linked to the 'Tardy' cluster class. This provided evidence that initial translation in the cycle might be directed from stable transcripts present in the infectious EB form. Based on these criteria the novel 'Tardy' class was separated from the 'Late' class. The gene ontologies were used to identify specific pathways and physiological functions active during the different phases of development. Additionally, the transcriptome

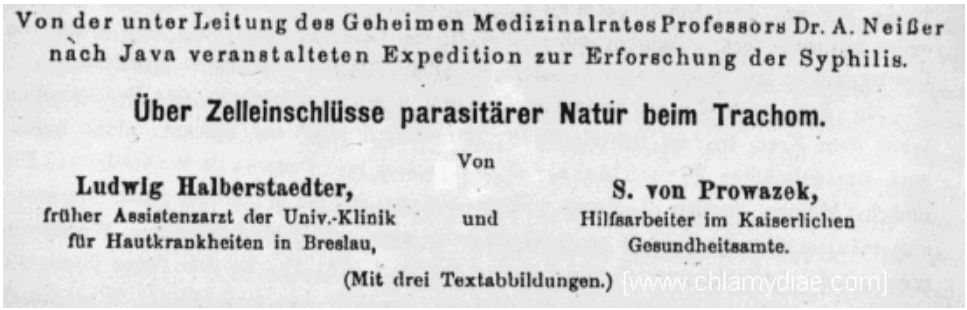
of *Cpn* in the persistent stage was compared to that of the acute developmental cycle. The *Cpn* transcriptome was altered in the iron-depletion mediated persistence. Genes up-regulated were linked to clusters at the beginning of the developmental cycle, and genes down-regulated were linked to clusters at the end of the developmental cycle. These data provided strong evidence that the *Cpn* transcriptome during persistence is a gene expression arrest in mid-development. In early acute infection convergently or divergently oriented gene pairs preferentially had an antagonistic expression profile, whereas tandemly oriented gene pairs showed a correlated expression profile. This suggests that the *Cpn* genome is organized mainly in tandemly arranged operons and in convergently or divergently oriented genes with favored antagonistic profiles. The microarray studies done with the *Cpn* strain *CWL029* also showed expression signals for several genes annotated only for the *Cpn* strains *AR39* and *J138*. BLAST comparison verified that these genes are also coded in the *CWL029* genome. Several of these genes were convergently arranged with their neighboring gene and shared overlapping genome information. Among these were *parB*, involved in DNA segregation and *rpsD,* an alternative sigma factor responsible for the transcription at late stages of the developmental cycle. Both genes have been described to have major roles in the chlamydial cycle. These genes had an antagonistic expression profile at the beginning of the acute developmental cycle and in persistence, as described before to be predominant for convergently oriented genes. Real time RT-PCR analysis showed that full-length *rpsD* mRNA transcripts were down-regulated, whereas short-length *rpsD* mRNA transcripts were up-regulated during the persistent infection. This demonstrated that the *rpsD* promoter is activated during the persistent infection and that because of the collision of the RNA polymerases full length transcripts were down-regulated. This sigma factor-independent mechanism is known as ‘Transcriptional Interference’. This is the first description on how the alternative sigma factor *rpsD* might be down-regulated during persistent infections. Finally, the host cell transcriptome was analyzed in the acute and persistent infection mediated by the depletion of iron. *Cpn* infection triggered the up-regulation of *relB*, involved in an alternative NF-κB signaling pathway. Several genes coding for cell cycle proteins were triggered, including *cyclin G2* and *cyclin D1* and inhibitors of CDK4. It can be assumed that *Cpn* infection leads to a G1-S transition in the host cell cycle. Interestingly the persistent infection had a stronger influence on prominent cell cycle genes. Several metabolite transporters were differentially expressed, with *aquaporin-7* only being down-regulated in the persistent infection. Overall *Cpn* triggers expression of host cell genes involved in anti-apoptosis, cell cycle and cell metabolism. Taken together, this work provides insights into the modulation of the pathogen and the host transcriptome during the acute infection and the iron mediated persistent infection.

Table of Contents

I Introduction

1.1 History of Chlamydiaceae

Descriptions of chlamydia-like diseases, resembling the symptoms of trachoma, already have been described in ancient Chinese and Egypt writings. 1907 Haberstädter and Prowazek published a work, performed on the island of Java, describing the transmission of conjunctivitis from men to orangutan (Figure 1). Using 'Giemsa' staining of epithelial cells they found intracellular vacuoles and smaller particles that have been correctly recognized as the cause of the trachoma.


Von der unter Leitung des Geheimen Medizinalrates Professors Dr. A. Neißer nach Java veranstalteten Expedition zur Erforschung der Syphilis.

Über Zelleinschlüsse parasitärer Natur beim Trachom.

Von

Ludwig Halberstaedter, früher Assistenzarzt der Univ.-Klinik für Hautkrankheiten in Breslau, und S. von Prowazek, Hilfsarbeiter im Kaiserlichen Gesundheitsamte.

(Mit drei Textabbildungen.) [www.chlamydiae.com]

Figure 1 - **The cover letter of the original publication from Halberstaedter and Prowazek from 1907.** This is the first scientific report of Chlamydiae (picture taken from www.chlamydiae.com).

Some of the particles were discovered outside the cell, most likely being elementary bodies (Figure 2). This was a remarkable triumph for the early times of infectious biology as these particles are about 0.4 µm in diameter, and only just above the 0.25 µm resolution limit for conventional light microscopy. Because of the blue staining matrix, the newly discovered particles were called *Chlamydozoa* (from the Greek khlamus, for mantle or cloak). Later, the assumption that they are "mantled protozoan" turned out to be incorrect, though the name survived. Similar inclusions were described in the conjunctival cells of babies with the non-gonococcal urethritis o*phthalmia neonatorum* acquired from their mothers and in the urethral epithelium from male patients. This pathogen is nowadays known as *Chlamydia trachomatis (Ctr).* The observations that growth is not possible in artificial medium as well as the property to cross bacteria dense membranes led to the incorrect conclusion that these particles would be viruses. From 1929 to 1930 frequent outbreaks of pneumonia adapted from birds, termed psittacosis, were observed. At this time, Levinthal, Coles und Lillie discovered particles in tissue from patients and Bedson described the characteristic developmental cycle. It took more than 30 years before the concept of an "obligatory intracellular parasite with bacterial capacities", long time referred to as *Bedsoniae*, to be accepted. 1935 *C. psittaci* and *C trachomatis* were propagated for the first time. The term Chlamydiae reappeared again in the literature in 1945. The discovery of bacterial RNA, ribosomes and cell wall structures in 1965 led to the conclusion that

Chlamydiae are not viruses. In 1990 Chlamydiae was described for the first time as an „emerging infectious disease causing pathogen“.

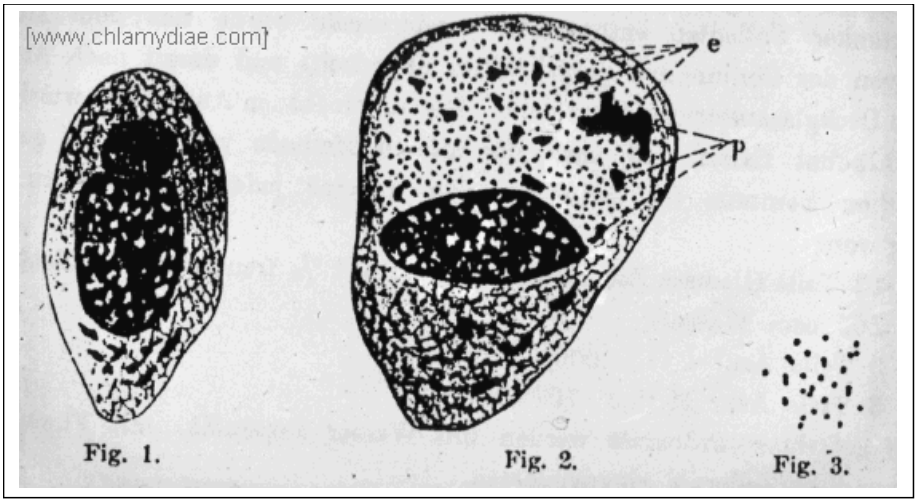


Figure 2 - **Drawings done by Halberstaedter and Prowazek** with an uninfected cell (left); an infected cell (middle) and from chlamydial particles (right) (Taken from www.chlamydiae.com).

1.2 The chlamydial taxonomy

Until 2000 the systematic subdivision of Chlamydiales has been mostly based on pheno- and genotypic features (Kaltenboeck, B. et al., 1993) (Figure 3). They were separated in a distinct group from the eubacteria primarily because of the existence of two membranes, one of the main features of gram-negative bacteria (Schachter, J. *et al.*, 1980). The only member of this order was the family Chlamydiaceae. The genus *Chlamydia* was separated into four different species, *Chlamydia pecorum*, *Chlamydia pneumoniae*, *Chlamydia psittaci* und *Chlamydia trachomatis.* In 2001 a new system of classification has been introduced, based on 16S rRNA and 23S rRNA sequence comparisons (Figure 3). Even though this classification is still a matter of debate, the Chlamydiaceae are now divided based on these rRNA analyses. The family Chlamydiaceae is subdivided into the two genera *Chlamydia* and *Chlamydophila.* Additionally the families Parachlamydiaceae, Waddliaceae and Simkaniaceae were introduced into the order of Chlamydiales (Everett, K. D. *et al.*, 1999;Schachter, J., 2001).

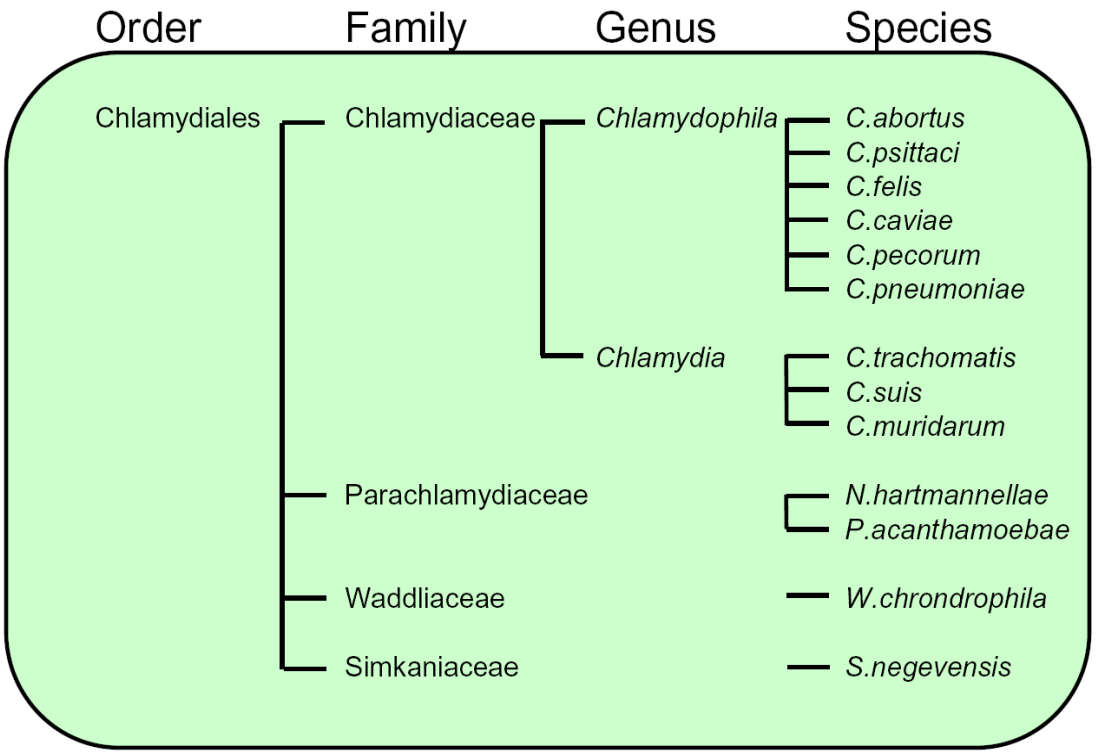


Figure 3 - **New taxonomy of the order Chlamydiales** (Adapted from: Bush and Everett, K.D.E.; 2001).

1.3 The chlamydial cycle of development

The most important characteristic feature of the family Chlamydiaceae is the biphasic cycle of development (Figure 4). This cycle alternates between the metabolic inactive, spore-like, extracellular, infectious elementary bodies (EB) (~ 0.3 µm) and the metabolically active but non-infectious reticulate bodies (RB) (~ 1 µm). Upon infection the metabolically inactive EB adhere to the host cell. The factors responsible for this adhesion process, neither for the host cell nor for the bacterium, have not yet been identified. However, the role of several chlamydial proteins, like Hsp70 or PmpD, in this process has been discussed (Raulston, J. E. *et al.*, 1993;Wehrl, W. *et al.*, 2004c). After attachment of the EB to the host cell the bacteria are internalized by phagocytosis or receptor-mediated endocytosis and uptake processes have been described to be mediated by clathrin dependent and independent pathways (Murray, A. *et al.*, 1984;Reynolds, D. J. *et al.*, 1990;Hodinka, R. L. *et al.*, 1988a;Schramm, N. *et al.*, 1995;Boleti, H. *et al.*, 1999;Norkin, L. C. *et al.*, 2001;Stuart, E. S. *et al.*, 2003;Carabeo, R. A. *et al.*, 2002b). The invading pathogen modifies phagosomal properties and prevents entry into the lysosomal pathway (Al Younes, H. M. *et al.*, 1999;Fields, K. A. *et al.*, 2002;Ojcius, D. M. *et al.*, 1997;van Ooij, C. *et al.*, 1997;Heinzen, R. A. *et al.*, 1996;Taraska, T. *et al.*, 1996). After successful entry the chlamydial EB inhabit a vacuole, the so-called inclusion (Sinai, A. P. *et al.*, 1997). Recently, a Type Three Secretion

System has been discovered in Chlamydiae (Kalman, S. *et al.*, 1999j). Characteristic markers of the chlamydial inclusion are the Inc-proteins, which are very likely to be secreted into the inclusion membrane via this Type Three Secretion System (TTSS) (Subtil, A. *et al.*, 2000;Subtil, A. *et al.*, 2001). As early as two hours after internalization the differentiation from EB to RB begins which is followed by initial mRNA expression (Fields, K. A. *et al.*, 2003). It has been shown that the movement of the chlamydial inclusion to the perinuclear region close to the microtubulin organizing center (MTOC) depends on active pathogen gene expression (Clausen, J. D. *et al.*, 1997;Grieshaber, S. S. *et al.*, 2003). Six to ten hours post infection (p.i.) the bacteria start multiplying by binary fission, also indicated by the increasing size of the inclusion. The chlamydial inclusion receives anterograde vesicular traffic from the golgi normally destined for the plasma membrane (Hackstadt, T. *et al.*, 1995;Wylie, J. L. *et al.*, 1997;van Ooij, C. *et al.*, 2000;Wolf, K. *et al.*, 2001b;Carabeo, R. A. *et al.*, 2003). Approximately 16 h to 20 h p.i. for *C. trachomatis* and 48 h p.i. for *C. pneumoniae* the re-differentiation process from RB to EB begins (Wolf, K. *et al.*, 2001a;Hodinka, R. L. *et al.*, 1988b;Wolf, K. *et al.*, 2000) and the developmental cycle is getting increasingly asymmetric. The signals for the initiation of RB to EB re-differentiation are still unknown. The chlamydial chromosome undergoes structural changes during this process which is primarily influenced by the histone-like protein Hc1 that has the capacity to condense DNA (Barry, C. E., III *et al.*, 1992;Barry, C. E., III *et al.*, 1993). With 72 h p.i. the re-differentiation process is mainly completed for *C. pneumoniae*. The EB are released into the environment by lysis of the host cell and neighboring cells are infected (Campbell, S. *et al.*, 1989;Rockey, D. D. *et al.*, 1996). The host cell itself is also influenced by the pathogen. The appearance of a Type Three Secretion System in the pathogen suggests that proteins secreted via the inclusion membrane influence host cell pathways (Kalman, S. *et al.*, 1999i). Chlamydia secrete a protease that has the capacity to digest the MHC-class-I transcription factor (Zhong, G. *et al.*, 2001b;Heuer, D. *et al.*, 2003c). Additionally apoptosis is blocked (Rajalingam, K. *et al.*, 2001;Fan, T. *et al.*, 1998b) and it has been shown that the activation of Bax and Bak, members of the Bcl-2 family of apoptosis-regulating proteins, is inhibited (Xiao, Y. et al., 2004). Furthermore the infection leads to a change in the cytoskeleton of the host cell and to the phosphorylation of host cell proteins (Kalman, S. *et al.*, 1999h;Birkelund, S. *et al.*, 1997;Fawaz, F. S. *et al.*, 1997b;Carabeo, R. A. *et al.*, 2002a).

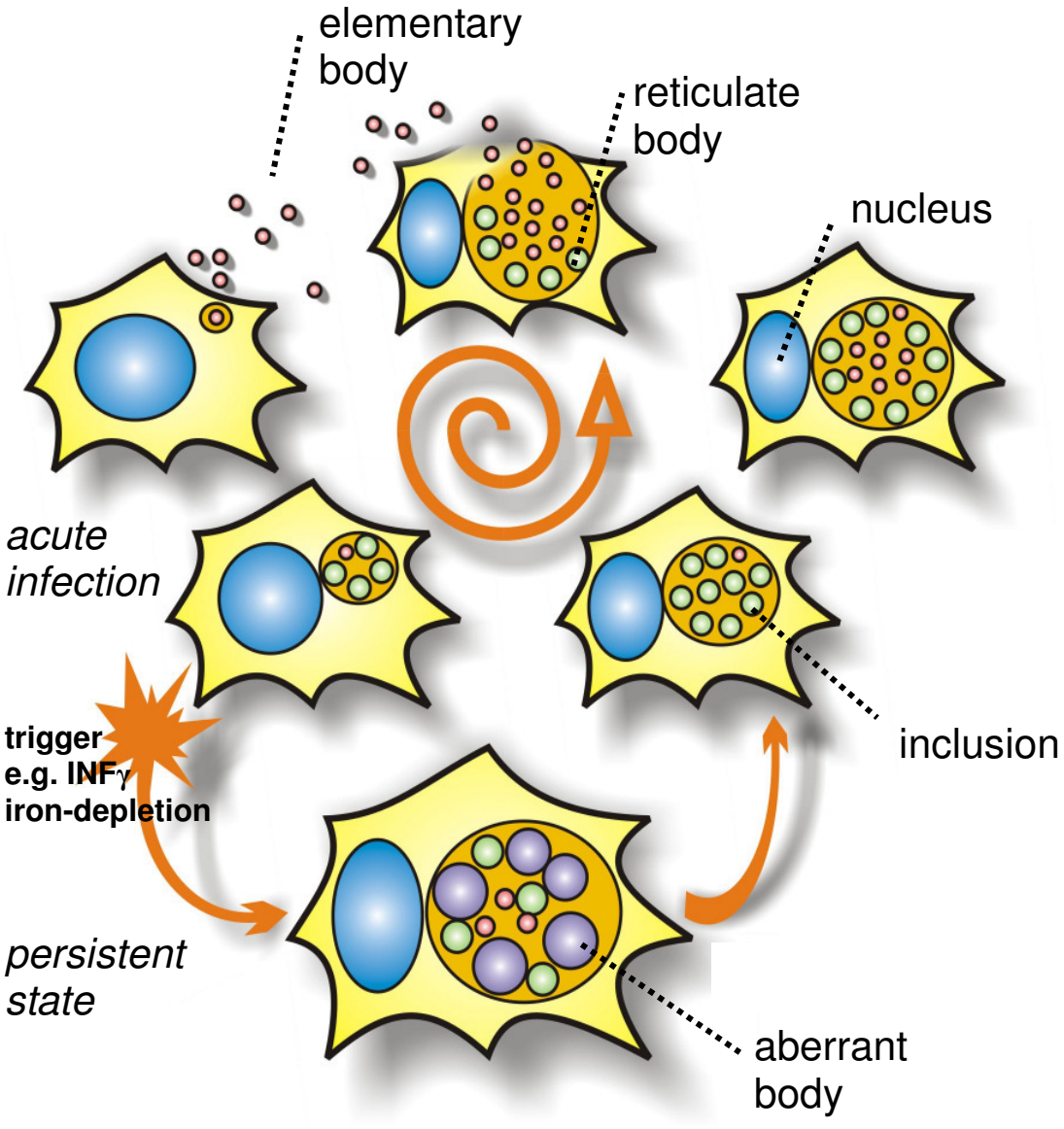


Figure 4 - **Developmental Cycle of *Chlamydophila pneumoniae.*** After uptake of the elementary bodies (EB) into the host cells the bacteria modify the residing vacuole, the inclusion. They differentiate to metabolic active reticulate bodies (RB) and multiply by binary fission. At the end of the developmental cycle the RB redifferentiate back to EB and are set free by host cell lysis to begin a new developmental cycle. Certain growth conditions, like treatment with INFγ or depletion of iron, lead to a persistent state, which can last for an indefinite time. Removing of the persistence stimulus leads to a continuation and completion of the developmental cycle *in vitro*.

Beside completing the developmental cycle Chlamydiae can also undergo a persistent infection. This persistent infection can be induced by different conditions, which inhibit the RB to EB re-differentiation process. As a result particles with a changed morphological appearance, so-called aberrant bodies (AB), develop (de la Maza, L. M. *et al.*, 1987b;de la Maza, L. M. *et al.*, 1987a). Persistent infections have the capacity to reside in the host organism for an indefinite time. The release of the stimulus can lead to a continuation of the developmental cycle followed by an initiation of the RB to EB re-differentiation *in vitro*.

1.4 The genome of *C. pneumoniae*

The sequencing of chlamydial genomes has been an important milestone for the work with this pathogen as there are no methods to directly manipulate their genome.
Comparison with genomes from other pathogens led to a variety of interesting findings: The assumption, that Chlamydiae rely exclusively on the import of host ATP has been counteracted (Kalman, S. *et al.*, 1999g;Stephens, R. S. *et al.*, 1998f;Read, T. D. *et al.*, 2000a), as the genome codes for several enzymes taking part in metabolic pathways. Therefore it can be assumed that Chlamydiae can in fact deliver a part of the energy on their own by synthesizing ATP or NADPH. Also genes coding for ATP/ADP translocases have been identified and it has been shown that Chlamydiae uptake ribonucleotidtriphosphate via specific transporters (Tjaden, J. et al., 1999). Furthermore, genome sequencing revealed that genes for the synthesis of peptidoglycan exist, even though the amount actually synthesized in EB appears to be very small. A whole superfamily of chlamydia specific proteins, the so-called polymorphic proteins (Pmp) was discovered and finally an unexpectedly high number of chlamydial genes is related to higher organisms, particularly plant chloroplasts. Moreover, the sequencing of chlamydial genomes opened the possibility to clone chlamydial genes easily and to use these data for expression studies especially microarray studies. Up to now, 10 different strains have been sequenced including *Chlamydia muridarum*, *Chlamydia trachomatis* A/HAR-13, D/UW-3/CX, *Chlamydophila abortus* S26/3, *Chlamydophila caviae* GPIC, *Chlamydophila pneumoniae AR39*, *CWL029*, *J138*, *TW-183* and *Parachlamydia* sp. UWE25, making this family to the most sequenced one. The strain *CWL029*, used in this study, possessing a circular genome of 1.23 Mb, was sequenced in 1999 at Stanford and UC-Berkeley and described in the key publication "Comparative Genomics of *Chlamydia pneumoniae* and *C. trachomatis*" (Kalman, S. *et al.*, 1999f).

1.5 Correlation between persistence and chronic diseases

A characteristic feature for all species in the family of Chlamydiaceae, but in particular for *C. pneumoniae*, is the potential to cause chronic diseases, which can last for years or even decades and therefore are very difficult to cure. Chronic *Cpn* infections are connected to a variety of respiratory and systemic diseases, like asthma or atherosclerosis (Pasternack, R. *et al.*, 2005;Ford, P. J. *et al.*, 2005). A possible explanation for the establishment of chronic diseases has been given by the discovery of chlamydial persistence *in vitro* (Hammerschlag, M. R., 2002a;Hammerschlag, M. R., 2002b;Hammerschlag, M. R., 2002c). Typical for the chlamydial persistence is an altered developmental cycle with smaller inclusions and less, but larger particles. These persistent particles are called aberrant bodies (AB) and possess a different metabolic activity (Shatkin, A. A. *et al.*, 1985). Because RB to EB re-differentiation does not take place and of the absent cell lysis these persistent infections are not productive (Beatty, W. L. *et al.*, 1995;Gerard, H. C. *et al.*, 2002a;Mathews, S. *et al.*, 2001;Byrne, G. I., 2001;Byrne, G. I. *et al.*, 2001a;Belland, R. J. *et al.*, 2003c). The possibility of reactivating the developmental cycle *in vivo* has been discussed and demonstrated also to take place during the process of immunosuppression (Malinverni, R. *et al.*, 1995;Yang, Y. S. *et al.*, 1983). Possible mechanisms of the persistent infection has been postulated on a molecular level: the amount of Hsp60, which has been described to be an immunostimulating protein, increases relatively to the total protein composition in a *C. pneumoniae* or *C. trachomatis* infected cell when treated with IFNγ (Beatty, W. L. *et al.*, 1993;Mehta, J. L. *et al.*, 1998). The same has been discovered for *in vivo* infections, where Hsp60 leads to hypersensitivity or autoimmune diseases (Gerard, H. C. *et al.*, 1998;Morrison, R. P. *et al.*, 1989a;Morrison, R. P. *et al.*, 1989b). *Cpn0483,* a hypothetical protein, has been observed to be up-regulated in chronic *C. pneumoniae* infections and is thought to play a role in the induction of autoimmune encephalomyelitis in rats (Lenz, D. C. *et al.*, 2001;Hogan, R. J. *et al.*, 2003c). Additionally, not only chlamydial gene products but also the host answer to the infection, especially the continuous production of inflammatory cytokines can cause long term effects and tissue damage triggered by persistent infections (Stephens, R. S., 2003;Ward, M. E., 1995).

1.6 *In vitro* induced persistence models

To study the molecular basics of persistence, different *in vitro* persistence models have been established. Infections of *C. pneumoniae (Cpn)* in fibroblast-like synovial cells or peripheral blood monocytic cells (PBMC) develop a spontaneously and irreversible persistence (Koehler, L. *et al.*, 1997;Airenne, S. *et al.*, 1999). A continuous infection model leads to a persistent phenotype for a part of the inclusions in HEp-2 and HeLa cells, the standard cell lines for acute infections (Kutlin, A. *et al.*, 2001a;Al Younes, H. M. *et al.*, 2001d) (Figure 5). Certain culture conditions, like amino acid or glucose deprivation and antibiotic treatment, also lead to persistent conditions (Kutlin, A. *et al.*, 2001b;Gieffers, J. *et al.*, 2004;Bin, X. X. *et al.*, 2000). Of special interest is the simulation of inflammatory conditions achieved through the addition of cytokines like IFNγ. or TNFα to the culture medium (Byrne, G. I. *et al.*, 1986;Summersgill, J. T. *et al.*, 1995a). These inflammatory cytokines lead to an induction of the Indolamine 2,3-Dioxigenase (IDO) resulting in the cleavage of the indolaminering of L-tryptophan and therefore to decreased concentration of amino acids in the host cell (Pantoja, L. G. *et al.*, 2000a). IFNγ und TNFα also lead to an up-regulation of the human trp-tRNA-synthetase in the host cell (Turpaev, K. T. *et al.*, 1996). Since human trp-tRNA cannot be used by the pathogen, this results in a decreased availability of tryptophan for the bacterium (Takikawa, O. *et al.*, 1988;Cheshire, J. L. *et al.*, 1997;Flohr, T. *et al.*, 1992). Additionally it has been shown that an excess of tryptophan in the cell culture medium counteracts the influence of IFNγ and allows *C. pneumoniae* to continue with the developmental cycle. The same has been discovered during the inhibition of IDO in smooth muscle cells of the aorta (Summersgill, J. T. *et al.*, 1995b;Shemer, J. *et al.*, 1989;Pantoja, L. G. *et al.*, 2000b). However, also additional explanations for the influence of IFNγ on the infection exist. It has been shown that the nitrogen monoxide (NO) synthetase is activated leading to the production of NO and other reactive oxygen species (ROS) which have bactericide properties. NO, a free radical, binds to iron atoms with a high affinity, leading to the inhibition of a variety of enzymes with iron as a cofactor (Igietseme, J. U. *et al.*, 1996;Mayer, S. M. *et al.*, 1993;Drapier, J. C. *et al.*, 1993). IFNγ also decreases the synthesis of the transferrin receptor, resulting in a reduction of the available iron in the cell and an inhibition of iron dependent processes (Byrd, T. F. *et al.*, 1993). Iron is involved in many vital cellular functions, such as electron transport, DNA synthesis and as a cofactor for many enzymes (Ponka, P., 1999) and is therefore necessary for bacterial survival. It was shown that the establishment of coronary artery infection and heart diseases, specifically from *Cpn*, only takes place in an excess of iron (Sullivan, J. L. *et al.*, 1999;Alpert, P. T., 2004) and fluctuating iron levels, e.g. under the influence of estradiol in endometrial tissues also contribute to the outcome of chlamydial infections (Kelver, M. E. et al., 1996). Limitation

of iron availability by up-regulated expression of the iron-binding proteins transferrin, ferritin and lactoferrin is part of the early host defense system against bacterial infections (Schaible, U. E. *et al.*, 2004;Weinberg, E. D., 1984a). This leads to a reduction of the free iron pool in the affected tissues to a level lower than bacteria need for their growth (Weinberg, E. D., 1984b;Ratledge, C. *et al.*, 2000). It has been shown that transferrin is up-regulated in host cells infected with *C. trachomatis* (Gail, M. et al., 2001) but on the other hand iron is essential for chlamydial growth (Ishihara, T. et al., 2005).

A reduction of iron in the growth medium, mediated by the iron chelator deferoxamine (DAM), induces persistence of Chlamydiae (Igietseme, J. U. *et al.*, 1998) and leads to an altered developmental cycle with the described persistent forms, the aberrant bodies (AB). Reactivation of the persistence can be achieved by removing the chelator resulting in a continuation of the developmental cycle (Al Younes, H. M. *et al.*, 2001c). Moreover, the genome sequence of *Cpn* revealed several open reading frames (ORF) encoding putative iron-requiring proteins (Stephens, R. S. *et al.*, 1998e), and an iron-limiting environment leads to a differential protein expression pattern (Wehrl, W. *et al.*, 2004d). Together this suggests a central role for iron, however the influence of iron on the infection process is still poorly understood.

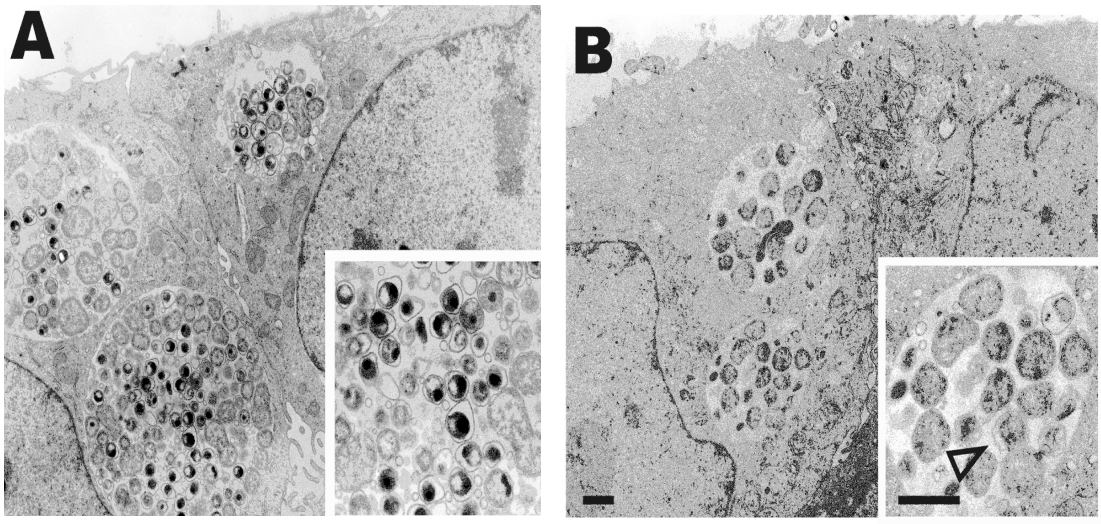


Figure 5 - **Electron microscopy of *C.pneumoniae* infected HEp-2 cells**. (A) Acute infection and (B) iron depletion persistent infection (Al Younes, H. M. *et al.*, 2001e). The arrowhead indicates the abberant forms.

1.7 Medical importance of human-pathogenic chlamydial strains

Even though the diversity of the chlamydial family is enormous, so far only three species have been connected to human diseases, including *C. trachomatis, C. pneumoniae* and *C. psittaci*.

Beside the two major human pathogens *C. trachomatis* and *C. pneumoniae* another strain from this family can be associated with a human disease. *C. psittaci* is the cause of ornithose or psittacosis (Peeling, R. W. *et al.*, 1996). In contrast to the described clinical symptoms of *C. trachomatis* and *C. pneumoniae,* the ornithose is a zoonose, mainly infecting birds. Nevertheless, in very seldom cases, e.g. when the animal – human contact is very close the pathogen can also transfer to humans. The clinical symptoms resemble an atypical pneumoniae that also can cause death of the patient.

C. trachomatis (Ctr) infections are among the most sexual transmitted diseases in the industrialized countries leading to infertility and untreated *Ctr* infections are the main cause of preventable blindness in developing countries. The transmission of the pathogen mostly takes place via smear infection. Two biovars have medical impact: the biovars trachoma and lymphogranuloma venereum (LGV) are causing different disease patterns in humans. These include trachoma, conjunctivitis, general infection of the genital tract and lymphogranuloma venereum. The two biovars are furthermore subdivided into the serovariants A to C leading to ocular infections; D to K, establishing infections of the urogenital tract and the serovariants L1 - L2 that are exclusively transmitted via sexual contact and lead to the establishment of lymphogranuloma venereum. The L-serovars have the capacity to spread to the lymph tissue what can result in necrosis and scars in the contaminated lymph knots. Moreover it has been discovered that infections with *Ctr* result in reactive arthritis in approximately 30% of all cases (Emre, U. *et al.*, 1995;Emre, U. *et al.*, 1994;Schachter, J. *et al.*, 1980).

C. pneumoniae (Cpn) has not been recognized as an independent pathogen before 1986. It is transmitted as an airborne disease (Dal Molin, G. *et al.*, 2005;Reechaipichitkul, W. *et al.*, 2005;Paldanius, M. *et al.*, 2005;Seuri, M. *et al.*, 2005;Janssens, J. P., 2005) and occurs worldwide with about half of the adult population having antibodies against *Cpn* (Grayston, J. T. *et al.*, 1990a). Since 70% of infections are subclinical or asymptomatic (Grayston, J. T. *et al.*, 1990b) many seropositive individuals are not aware that they have been infected with *Cpn*. The high prevalence of antibodies against *Cpn* suggests that infections often reappear (Grayston, J. T. *et al.*, 1990c;Aldous, M. B. *et al.*, 1992) but on the other hand there is evidence that *Cpn* often establishes persistent infections (Davidson, M. *et al.*, 1998;Falck, G. *et al.*, 1996;Hammerschlag, M. R. *et al.*, 1992). *In vivo*, persistence has been observed in blood monocytes (Bodetti, T. J. *et al.*, 2000;Boman, J. *et al.*, 2000a;Maass, M. *et al.*, 2000). The pathogen first infects the epithelium of the upper and lower part of the

respiratory tract (Creer, D. D. *et al.*, 2005). While most of the infections are unapparent, a minority manifests as pharyngitis, bronchitis or pneumonia, with symptoms lasting from weeks to months (Cunha, B. A., 1998). Along with chronic infections caused by *Cpn* Chronic Obstructive Pulmonary Disease (COPD), bronchial asthma, erythema nodosum[1], Guillain-Barré syndrome[2] and pulmonary sarcoidosis[3] have been observed (Kuo, C. C. *et al.*, 1995;Murphy, T. F. *et al.*, 2002). In recent years also a connection between chronic *Cpn* infections and diseases like arthritis, atherosclerosis, Alzheimer's disease, lung cancer, Sézary T-cell lymphoma or multiple sclerosis have been discussed (Balin, B. J. *et al.*, 1998;Itzhaki, R. F. *et al.*, 2004;Little, C. S. *et al.*, 2004;Balin, B. J. *et al.*, 2001;Abrams, J. T. *et al.*, 2001;Abrams, J. T. *et al.*, 1999;Hammerschlag, M. R. *et al.*, 2005;Hammerschlag, M. R. *et al.*, 2000;Ke, Z. *et al.*, 2000;Boman, J. *et al.*, 2000b;Belland, R. J. *et al.*, 2004). Nevertheless, the possible association of *Cpn* infections with these latter diseases is insufficiently understood and therefore still in discussion.

[1] *Erythema nodosum is a type of skin inflammation that is located in a certain portion of the fatty layer of skin. Erythema nodosum results in reddish, painful, tender lumps most commonly located in the front of the legs below the knees. The tender lumps, or nodules, of erythema nodosum range in size from 1 to 5 centimeters. The nodular swelling is caused by a special pattern of inflammation in the fatty layer of skin (from www.medicinenet.com).*

[2] *A disorder characterized by progressive symmetrical paralysis and loss of reflexes, usually beginning in the legs. The paralysis characteristically involves more than one limb (most commonly the legs), is progressive, and usually proceeds from the end of an extremity toward the torso. Areflexia (loss of reflexes) or hyporeflexia (diminution of reflexes) may occur in the legs and arms. Guillain-Barre syndrome is not associated with fever, an important fact in differentiating Guillain-Barré from other diseases. Guillain-Barré usually occurs after a respiratory infection, and it is apparently caused by a misdirected immune response that results in the direct destruction of the myelin sheath surrounding the peripheral nerves or of the axon of the nerve itself. The syndrome sometimes follows other triggering events, including vaccinations (from www.medterms.com).*

[3] *Sarcoidosis is a rare disease that results from inflammation. Ninety percent of the cases of sarcoidosis are found in the lungs, but it can occur in almost any organ. It causes small lumps, or granulomas, which generally heal and disappear on their own. However, for those granulomas that do not heal, the tissue can remain inflamed and become scarred, or fibrotic. Pulmonary sarcoidosis can develop into pulmonary fibrosis, which distorts the structure of the lungs and can interfere with breathing. Bronchiectasis, a lung disease in which pockets form in the air tubes of the lung and become sites for infection, can also occur (from www.medterms.com).*

1.8 Introduction into the Microarray Technology

Microarray analysis has emerged in the last few years as a flexible method for analyzing large numbers of nucleic acid fragments in parallel.

Despite the variety of technical solutions that have been developed for performing microarray analysis, all are miniaturized hybridization assays for studying thousands of nucleic acid fragments simultaneously. All microarray systems (Figure 6) share the following key components:

- the array, containing immobilized nucleic acid sequences,
- one or more labeled samples or 'probes',
- quantification of the hybridization signals.

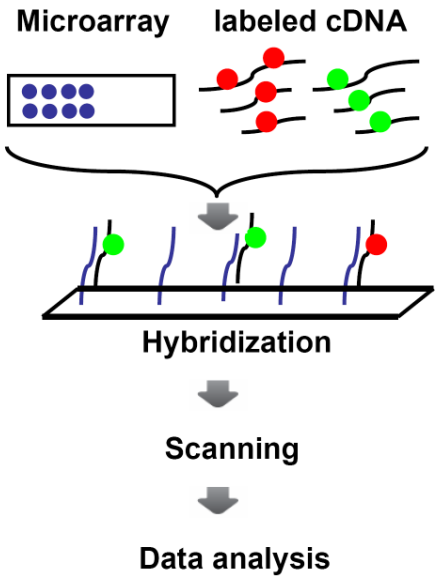


Figure 6 - **Principles of Microarrays.** Labeling, hybridization, scanning and data mining. Two samples (here sample red and green) are labeled, each with a nucleotide coupled to a different Cydye (mainly Cy3 or Cy5). Both are pipetted onto a microarray giving the typical two color hybridization.

For eukaryotes a variety of techniques exists to amplify mRNA out of the total RNA pool. The most popular procedure is based on the usage of the poly-A-tail (Figure 7). For prokaryotic RNA, not possessing a poly-A-tail, this labeling procedure is not possible. The procedure used in this work is based on the usage of an aminoallyl base to which in a second step the fluorescent dyes are coupled (Figure 8).

The labeled fragments in the probe are expected to form duplexes with their immobilized complementary targets. By measuring the different fluorescent signals associated with each spot, the relative abundance of specific sequences in each of the samples can be determined.

Microarray studies aim to identify genes or classes of genes that are differentially regulated across different classes of samples. In this case it is important to state the correct degree of evidence: the 'p-value'. A few microarray publications still present p-values as if each gene had been tested independently, even though many genes were actually tested in parallel. These p-values are wrong in the context of testing thousands of genes. A way to specify the confidence of microarray results is the 'false discovery rate' (FDR) (Klipper-Aurbach, Y. *et al.*, 1995;Hochberg, Y. *et al.*, 1990). This approach is based on permutation testing this approach is particularly useful for microarray studies because it can be easily adapted to estimate significance levels for many genes in parallel. Recent software packages, notably the Significance Analysis of Microarrays (SAM) (Tusher, V. G. *et al.*, 2001), implement this permutation based testing method.

The meaning of a p-value from a permutation procedure differs from the meaning of a model based p-value. The model based p-value is the probability of the test statistic[4], assuming that the gene levels in both the treatment and control groups follow the model (e.g. a Normal distribution). A test statistic is a quantity calculated from the sample of data. Its value is used to decide whether or not the null hypothesis should be rejected. A permutation based p-value tells how rare that test statistic is, among all the random partitions of the actual samples. The p-value for the gene is the fraction of cases in which the randomly permuted samples give a test statistic for that gene, at least as extreme as the one that occurs in the properly labeled samples. The idea is that if the gene is distributed similarly in both treatment and control groups, then the statistical difference (a t-statistic or any other) will appear about as significant in the permutated arrangement, as in the true arrangement. If the gene levels in the treatment group are higher than any levels in the control group, then no value of the permutated statistic will be as significant as the true value.

[4] A test statistic is a quantity calculated from the sample of data. Its value is used to decide whether or not the null hypothesis should be rejected in the hypothesis test. The choice of a test statistic will depend on the assumed probability model and the hypotheses under question (from: http://www.cas.lancs.ac.uk/glossary_v1.1/hyptest.html#ts).

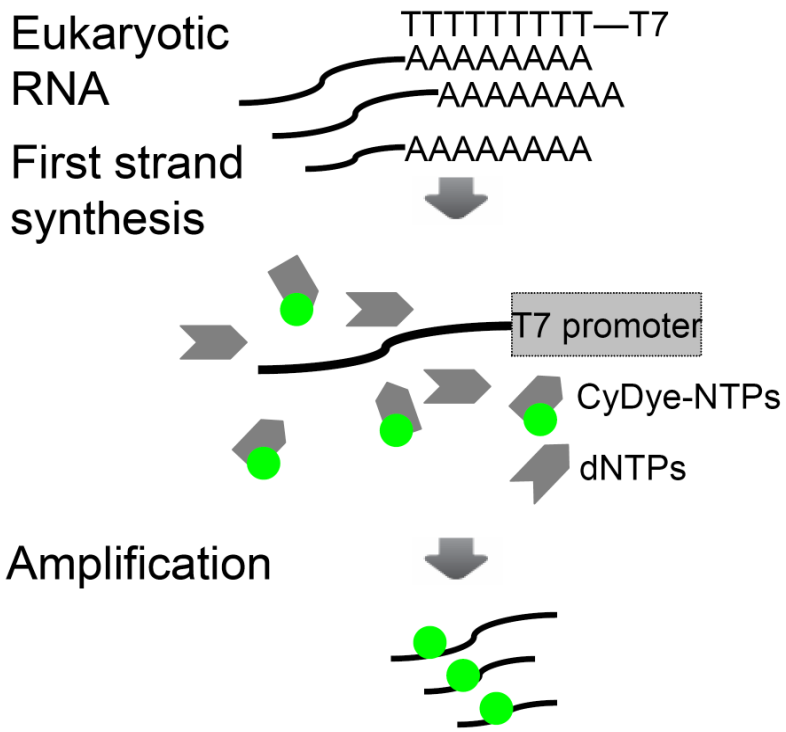


Figure 7 - **Amplification of eukaryotic mRNA**. Amplification of eukaryotic mRNA out of the total RNA pool. A poly-T primer with an attached T7 promoter binds to the poly-A-tail of the eukaryotic mRNA. The mRNA gets reverse transcribed in a 1st step and amplified and labeled in a 2nd step with the use of a T7 polymerase.

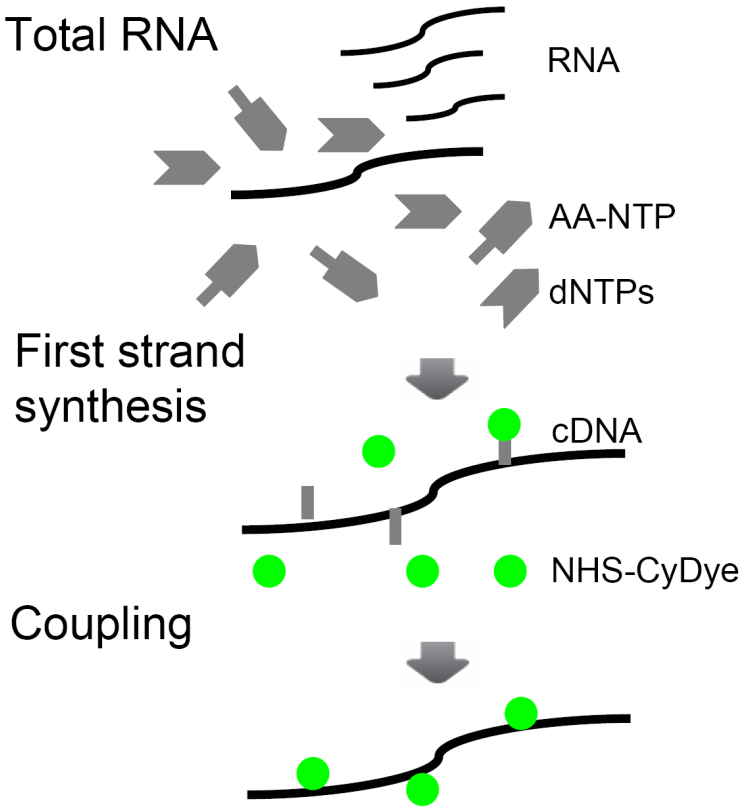


Figure 8 - **Aminoallyl (AA) labeling for prokaryotic RNA.** After purification, the RNA is reverse transcribed using an AA base. In a second step a special NHS ester Cydye is coupled to this AA base. Uncoupled NHS ester Cydyes are bound with a quencher.

1.9 Aim of this project

C. pneumoniae (*Cpn*) is the causative agent of diseases of the upper and lower respiratory tract, namely pneumoniae, bronchitis and sinusitis. Furthermore, *Cpn* infections are associated with severe chronic diseases like asthma, COPD, multiple sclerosis or coronary artery disease. So far, only little is known about the principle processes throughout the developmental cycle and during persistent infections. Especially persistent infections have been linked to these chronic diseases mentioned above and several models are used for *in vitro* studies of *Cpn* persistence, especially IFNγ stimulation, antibiotic treatment and iron depletion. The latter mimics the body's potential to limit bacterial infections by iron withdrawal. This is an important host mechanism to fight bacterial infections. How bacteria react to iron-limiting conditions is of enormous interest. Recently, studies characterized the *C. trachomatis* genome in the acute infection and the IFNγ persistence model. For *Cpn* only a limited number of publications addressed gene expression using RT-PCR. The aim of this study was to investigate the *Cpn* transcriptome in acute as well as iron depletion-mediated persistent conditions. To this end, the goal was to develop and validate a *Cpn* specific microarray in cooperation with MWG AG (Ebersberg, Germany). Also the protocols for RNA extraction, labeling and hybridization had to be established, as standard protocols could not be used for this obligate intracellular pathogen. Following the establishment of the microarray methods, the pathogen's transcriptome throughout the developmental cycle was to be analyzed. The obtained data was analyzed and significant gene expression characterized. Furthermore, the *Cpn* transcriptome in the iron depletion-mediated persistence was compared to that of the acute developmental cycle. Additionally, comparison of the gene expression of the acute and persistent infection was thought to give an insight into mechanisms of gene regulation important for the acute developmental cycle and the establishment of the *Cpn* persistence. Finally, also the host transcriptome was to be analyzed clarifying the impact of the acute *Cpn* infection and the iron mediated persistent infection on the host cell.

II Materials and Methods

2.1 Materials

2.1.1 Bacterial isolates

C. pneumoniae strain *CWL029* (*VR1310*) obtained from Washington Research Foundation (WRF).

2.1.2 Eukaryotic host cells

HEp-2 human epithelial cells originated from laryngeal carcinoma (ATCC-CCL23) obtained from the American Type Cell Collection (ATTC).

2.1.3 Medium for chlamydial propagation

Propagation medium was used for the propagation of chlamydial stocks and was composed of (Iscove's Modified Dulbecco's Media) IMDM medium (SIMGA, Germany) supplemented with 5% fetal bovine serum (FBS), 1% nonessential AA, 8.8% glucose and 10 µg gentamicin per ml. Additionally 1 µg cycloheximide per ml was added.

2.1.4 Maintenance medium

Maintenance medium (MM) was used for the maintenance of the Hep-2 host cells and consisted of RPMI medium with HEPES and 10% FBS. Additionally 10 µg of gentamicin per ml were used. MM was used during cell cultivation.

2.1.5 Infection medium

Infection medium (IM) was used for performing the infection experiments and consisted of RPMI with HEPES (SIGMA, Germany) and 5% FBS. In addition 1 µg cycloheximide per ml was included in the IM which was utilized in the acute and iron depletion experiments targeting the transcriptome of *C. pneumoniae*. For experiments targeting the transcriptome of the host cell no cycloheximide was added.

2.1.6 Primers

Table 1 – **Primers used in this study.**

Name	*Cpn#*	Forward	Reverse
lrcH1	*811*	CTCAAGGGAAGTACAACGAAGC	ATTGTTAGATTCTTCGGGTTGC
yscC	*702*	AAGAAGTCGAAGAGCCTGTACC	TCTCTGTAACTGTGGAGGTTTGC
sctS	*824*	ACCTCCCATCATCTTAGCTTCC	CGACTAGTTTGACTGCAAAAGC
sctN	*707*	AAAGCCGTAGTTCCTAATGTGC	TTCCTGCTCGAATGTGTAAGG
pmp18	*471*	GCCAGCTTTATGGTGATTGG	TTTTGATGCACGAAGAGTCG
groEL3	*898*	CCTTAAAGACGCGACAATTAGC	AATCTTAAGAGCGAGGGTATATAAAGG
16S	*16S*	CCAGTTCGGATTGTAGTCTGC	AGTCATCAGCCTCACCTTGG
minD	*805*	GTTTCATCCAAGGAATTTCAGC	ACAGGTTTGCCATGAATCG
murB	*988*	AGGAGGCTCGTGAAGTTATCC	CTAAAGCTGCAAAGGAAAGACC
cdp	*880*	CTTCCTAAGCCTTCGGTTCC	AGAACCGGGGATCTCTTCC
rs2	*696*	CGTCGATACTAACTGCGATCC	CGTCAGATTCGTCATCTTGG
tufA	*74*	GAAGGCGGACGTCATAAGC	GACCACCTTCACGAATTGC
L29	*639*	AGCGACGACGATTTAGATGC	TATGAACTTTCACAACTTTGTTTTGC
ABCT	*691*	GGTATCTTCGGGTGAGATTGC	CTCTGGAGGCATTTGAAAACC
htrA	*979*	CCTTCGGTCTTCAAGCTACG	AGGCTAGGAATCGCAAACC
nth	*837*	ACACTCAACGCGCTATTTCC	AAAATAGATTGGGCGTCTGG
secA2	*841*	CGCCATGATGTCTTACATGC	CAGCAGCGATCTTTTCAGC
gyrB2	*715*	ATGTAAAGGAAGCGATTGTACAGG	CCCTTCGGTAAGGAAAATCG
secA1	*260*	ACAGAAAGCGGCTGTTGG	ATATGCGAAAATCTTCTTGTGTAGG
gyrB1	*275*	TTCTACCGCGCTTACTAGGG	GTTTGCCCTTCAAATTGTGG
hctA	*886*	GATAGCATCCAACACGACTTAGC	GCAGGAGCCTTAGTTGATGG
hctB	*384*	AAACCTGCAGTTCGTAAGACG	GCTTTCTTAGGAGAACCTTTAGCC
omcB	*557*	GCGAAGCTGAATTCGTAAGC	TTGACCGCATTTAGTATAAGAACG
xseA	*1062*	TTTGGTTTACGCTGGAGAGG	AGTTCCCTTGGACAGTGACG
sctS	*824*	ACCTCCCATCATCTTAGCTTCC	CGACTAGTTTGACTGCAAAAGC
sycE	*325*	TTTAATCCGTTTCAGCGATACC	GTTGAGTGACCTCACCGTAGC
rpsD	*362*	CTCAATATTTCGCAACAAGAGC	AAGTTCCTGAATCGCATTGG
rpoD	*756*	TCTCTACAAGCCGAGGTTGG	AAACAAATCGCTCACGATCC
rpoN	*771*	TCCCTCTAAAGCACCTCTTCC	TTAGTTGTGCACGGTATTTTGC
IGR293	*IGR293*	AAGCTTATATGCAAGGCACCA	AGCAAAGGAATTATTCTCAGCGTA
CP0396-(A+)	*CP0396*	GGTCGCTATGTCTACATCTCCAA	CGTTCGGCTAGGGTAGAGGT
CP0396-(B+)	*CP0396*	GGTCGCTATGTCTACATCTCCAA	GCAATTGTGAAGATTGCTCCT
CP0396-(C+)	*CP0396*	GGTCGCTATGTCTACATCTCCAA	GAAAAGGAACGCAAGGTCAT
rpsD-(A-)	*CPn0362*	TGTGAAAACACAGCAAACTCAA	GCATCCCTGAAATCAAACG
rpsD-(B-)	*CPn0362*	TGTGAAAACACAGCAAACTCAA	CACCCGAAGCATACAAATCC
rpsD-(C-)	*CPn0362*	TGTGAAAACACAGCAAACTCAA	TGGCGAAGAGAATCCATAGC

2.1.7 Buffers and Solutions

PBS Dulbecco's	without Ca^{2+}, Mg^{2+} and $NaHCO_3$, sterile
Cycloheximide	0.1 g cycloheximide was dissolved in 2 ml absolute ethanol, aliquoted and stored at minus 20°C
DAM stock solution (3 mM)	0.1 g DAM (deferoxamine) was dissolved in 50.75 ml PBS, sterile-filtered, aliquoted and frozen at minus 20°C
Trypsin solution	0.05% (w/v) trypsin, 0.02% (w/v) EDTA in PBS; pH 7.2
SPG (cryopreserving buffer)	75 g sucrose, 0.52 g KH_2PO_4, 1.22 g Na_2HPO_4 and 0.72 g L-glutamic acid were dissolved in 1 l distilled H_2O. pH was then adjusted to 7.4 and solution was sterile-filtered and stored at 4°C
30% (v/v) Percoll	in 10 mM HEPES-145 mM NaCl; pH 7.4, stored at 4°C
PBS	0.2 g KCl, 0.2 g KH_2PO_4, 8 g NaCl, 1.15 g Na_2HPO_4 in 1 l distilled H_2O
Blocking and permeabilizing solution	0.2% (w/v) Triton X-100, 0.2% (w/v) BSA in PBS; pH 7.4
Fixative (4% (w/v) PFA-120 mM sucrose)	8 g PFA were dissolved in 120 ml distilled H_2O by heating at 60 – 70°C. Few drops of 1 N NaOH were added until clearance, followed by adding 20 ml of 10x PBS and 8 g sucrose and volume was then completed to 200 ml with H_2O. After cooling, pH was adjusted to 7.4 and the solution was aliquoted and frozen at minus 20°C

2.2 Methods

2.2.1 Eukaryotic host cells

The human epithelial HEp-2 cell line (ATCC-CCL23) derived from a laryngeal carcinoma was grown in RPMI medium (Gibco, Germany) with 10% FBS (Biochrome, Germany) at 37°C and 5% CO_2. Cells were regularly checked for mycoplasma contamination using nested PCR.

2.2.2 Propagation and infection

Cpn strain *CWL029* (ATCC VR1310) was propagated in HEp-2 cells. Cells were infected with a multiplicity of infection (MOI) of 2 in IMDM medium without supplementations, centrifuged at 37°C for 1 h at 900 x *g* , incubated at 35°C and 5% CO_2 for 1 h. Afterwards medium was replaced by IMDM medium as explained before supplemented with 5% FBS, containing cycloheximide (chex) (1 µg/ml). Cultures were incubated for 72 h at 35°C, harvested using glass beads (Sigma, Germany) and the supernatant was transferred to a 50 ml tube (Greiner, Germany). Host cells were lysed using glass beads (Sigma, Germany) centrifuged at 500 x *g* for 10 min to pellet cell debris. The supernatant was centrifuged at 12,000 x *g* for 1 h to pellet the EB. The pellet was washed with sucrose-phosphate-glutamate buffer (SPG, pH 7.4), recentrifuged and resolved in SPG-buffer, aliquoted and stored at minus 80°C. Stocks were determined to be free of mycoplasma contamination.

2.2.3 Infection for prokaryotic transcriptome studies

For acute and persistent infections HEp-2 cells were infected with *Cpn* at a multiplicity of infection (MOI) of 40 for 6 h and 12 h post infection (p.i.) and an MOI of 15 for other time points. Time course started upon infection (Fig. 1). To generate persistent infections 30 µM of deferoxamine (DAM) was added to the media throughout the infection process starting at the time of infection. Cells were incubated at 35°C and 5% CO_2 until the respective time point, washed with prewarmed PBS and harvested with 1 ml TRIzol (Invitrogen, Germany) per well (1 x 10^6 cells) and RNA was extracted following the prokaryotic RNA extraction protocol. The bacteria were examined by staining cell cultures grown on coverslips with an anti-chlamydial-LPS antibody (Progen, Germany) to confirm proper growth and establishment of acute and persistent infections.

2.2.4 Infection for eukaryotic transcriptome studies

Prior to infection, the EB progeny was cleaned from eukaryotic fractions. Purification was done as described by Mukhopadhyay (Mukhopadhyay et al., 2004), with slight modifications. Briefly, 10^8 cells were lysed in 5 ml NP-40 (Fluka) complemented SPG [1% NP-40 per 10^7 cells] and were subjected to sonication for 5 min followed by a 20 min incubation step with DNaseI [1.6 µg/ml] (Grade II, Roche) on ice. Nuclei and bigger fragments were removed by a 5 min centrifugation step at 4000 rpm. Post nuclear supernatant was layered on top of a 7 ml 50% to 25 ml 20% Ficoll-400 (Pharmacia) step gradient and was centrifuged at 18,500 g for 1 h at 4°C in a swing out rotor (Beckmann SW32TI). Interphase was collected and washed by addition of 10 ml SPG and centrifugation at 18,500 rpm for 30 min. Pellet was resupended by syringe and 5 min sonication to separate EB and suspension was aliquoted and stored at minus 70°C for further use. Purity was assessed by electron microscopy on a Leo 906E Transmission Electron Microscope.

For acute and persistent infections HEp-2 cells were infected with *Cpn* at a multiplicity of infection (MOI) of 2-3. Time course started upon infection. To generate persistent infections 30 µM of deferoxamine (DAM) was added to the media throughout the infection process starting at the time of infection. Cells were incubated at 35°C and 5% CO_2 until the respective time point, washed with prewarmed PBS and harvested with 1 ml TRIzol (Invitrogen, Germany) per well and RNA was extracted following the RNA extraction protocol for eukaryotic RNA. The bacteria were examined by staining cell cultures grown on coverslips with an anti-chlamydial-LPS antibody (Progen, Germany) to confirm proper growth and establishment of acute and persistent infections.

2.2.5 RNA Isolation, labeling and quality control for the pathogen array

Cpn infected HEp-2 cells were lysed with TRIzol. Total RNA was isolated with the following modified protocol: Cells were lysed with 1 ml Trizol per well (1 x 10^6 cells), transferred to a 2 ml reaction tube and sonicated for 5 min in a sonifier water bath. 0.2 ml chloroform was added per ml TRIzol. The mixture was shaken vigorously for at least 30 s, incubated 5 min at RT and centrifuged for 15 min at 12,000 x *g* at 4°C. The aqueous phase was transferred into a new tube. 0.5 ml isopropyl alcohol, 0.12 ml 5 M ammonium acetate and 10 µl glycogen (0.5 mg/µl) were added and mixed. The mixture was incubated 20 min at minus 20°C, centrifuged for 30 min at 12,000 x *g* at 4°C. The pellet was washed with 75% EtOH and resuspended in 85 µl H_2O. Additionally a DNase digest was performed according to the

manufacturers protocol (Quiagen, Germany). After the DNA digest a phenol/chloroform/isoamyl alcohol (25:24:1) extraction was performed. 0.1 v/v 3 M sodium acetate (pH 5.5) and 2.0 v/v EtOH were added and incubated at minus 20°C overnight, centrifuged at 12,000 x *g* at 4°C and resuspended in H_2O.

Integrity of the total RNA was verified with the Bioanalyzer 2100 (Agilent, USA) and RNA quantification was measured by photometer and Nanodrop (Kisker,Germany) prior labeling. For 6 h p.i. and 12 h p.i. 50 µg total RNA - and also 20 µg of the 48 h p.i. common control to avoid kit-specific biases - additionally was purified using the Microbeenrich™ (Ambion, USA) procedure to increase signal intensities.

A modified protocol for indirect incorporation of aminoallyl nucleotides during a first-strand reverse transcription reaction was established to yield a maximum of labeled cDNA copies of the total RNA pool for *Cpn*. Each labeling reaction consisted of 20 µg of total RNA (1 µg/µl) (or the yield of 50 µg total RNA processed with the Microbeenrich™ procedure), 2 µg random nonamer primers (Amersham, USA), 4.0 ml of dNTP master mix, and 2 µl (4 units/µl) of Superscript II reverse transcriptase (Stratagene). RNA and primers were heated to 65°C for 10 min, incubated at room temperature and put on ice 5 min before the remaining components were added. The reverse transcriptase reaction proceeded 2 h at 42°C. RNA was degraded by adding 10 µl (2.5 M) NaOH, incubating at 37°C for 15 min and neutralizing with 20 µl (2 M) HEPES. cDNA was precipitated by adding 6 µl (3 M) sodiumacetate, 150 µl EtOH, incubated at minus 20°C overnight and pelleted with 16,000 x *g* at 4°C. The pellet was resuspended in 0.1 M sodiumbicarbonate buffer (pH 9.0), added to either Cy 3 or Cy 5 mono NHS esters (Amersham), incubated at room temperature for 90 min and purified using 30K Microcon™ spin columns (Millipore, USA). The elution volume was diluted using hybridization buffer (Ambion) and quantified at A_{552nm} for Cy 3 and A_{650nm} for Cy 5 with a Nanodrop photometer (Kisker, Steinfurt, Germany).

2.2.6 RNA Isolation, labeling and quality control for the eukaryotic array

To extract RNA from infected and uninfected host cells *Cpn* infected HEp-2 cells were lysed with TRIzol. Total RNA was isolated with the following modified protocol: cells were lysed with 1 ml Trizol per well (10^6 cells), transferred to a 2 ml reaction tube and sonicated for 5 min in a sonifier water bath. 0.2 ml chloroform was added per ml TRIzol. The mixture was shaken vigorously for at least 30 s, incubated 5 min at RT and centrifuged for 15 min at 12,000 x g at 4°C. The aqueous phase was transferred into a new tube. 0.5 ml isopropyl alcohol, 0.12 ml 5 M ammonium acetate and 10 µl glycogen (0.5 mg/µl) were added and

mixed. The mixture was incubated 20 min at minus 20°C, centrifuged for 30 min at 12,000 x g at 4°C. The pellet was washed with 75% EtOH and resuspended in 85 µl H_2O.
Additionally the RNA was cleaned with the RNAeasy Kit (Quiagen, Germany) according to the manufacturer's instruction.
Microarray experiments were carried out as two-color dye-reversal ratio hybridizations on custom tailored 'in situ' microarrays encoding 8014 human genes (AMADID 010646, Agilent Technologies, Palo Alto, CA, USA) in biological duplicates. RNA labeling was performed with a Fluorescent Linear Amplification Kit (Agilent Technologies). In brief, cDNA was reverse transcribed from 4 µg total RNA with an oligo-dT-T7 promoter primer and MMLV-RT. Second strand synthesis was carried out with random hexamers. Fluorescent antisense cRNA was synthesized with either cyanine 3-CTP (Cy3-CTP) or cyanine 5-CTP (Cy5-CTP) and T7 polymerase. The fluorescent-labeled antisense cRNA was precipitated over night with LiCl, ethanol washed and resuspended in water. (In detail: to transcribe the RNA into T7 promoter-cDNA, 3 to 5 µg total RNA were mixed with 5.0 µl T7 promoter primer and incubated at 56°C for 10 min following 5 min at 4°C. The total RNA was mixed with 5x First Strand Buffer, 0,1 M DTT, 10 mM dNTP Mix, random hexamers, MMLV RT, RNase Out and 0,15% Triton X-102 according to the manufacturer, incubated at 40°C for 4 h, 15 min at 65°C and 5 min at 4°C. To label, the cDNA was incubated with 4 x transcription buffer, 0,1M DTT, NTP Mix, RNase Out (Invotrogen, Germany), inorganic pyrophosphatase and T7 RNA polymerase. Additionally Cy3 or Cy5 nucleotides were incubated with the mix. The mixture was incubated for 2 h at 40°C and 5 min at 4°C. 80 µl LiCl (4M) were added and incubated at minus 20°C overnight.) The purified products were quantified at A_{552nm} for Cy3-CTP and A_{650nm} for Cy5-CTP and labeling efficiency was verified with a Nanodrop photometer (Kisker, Steinfurt, Germany). Before hybridization, 1.25 µg labeled cRNA of each product were fragmented and mixed with control targets and hybridization buffer according to the supplier's protocol (Agilent Technologies). Hybridizations were done over night for approximately 17 h at 60°C. The slides were washed according to the manufacturer's manual and scanning of microarrays was performed with 5 µm resolution using a DNA microarray laser scanner (Agilent Technologies). In order to compensate dye specific effects, and to ensure statistically relevant data, a color swap dye reversal was performed.

2.2.7 Array design, hybridization and scanning for the pathogen array

A 50mer oligo array containing all open reading frames of the strains *CWL029* (http://www.stdgen.lanl.gov), *J138* (http://kantaro.grt.kyushu-u.ac.jp) and *AR39* (garnet.Berkeley.edu) was designed. To ensure a maximum specifity, oligo sequences were

blasted against the human genome. Design and spotting was done by MWG-Biotech AG (Germany). Cross hybridization of eukaryotic RNA was confirmed to be marginal by labeling and hybridizing eukaryotic total RNA with the described procedure. Arrays were blocked prior to hybridization by incubation for 45 min at 42°C in blocking solution (4x SSC, 0.5% SDS, 1% BSA), washed five times with H_20 and dried by centrifugation. Microarray experiments were done as two-color hybridizations. In order to compensate dye specific effects and to ensure statistically relevant data, a color swap was performed. Reverse transcribed cDNA pools were mixed with hybridization buffer (Ambion), denatured, snap-cooled, pipetted onto the array using Lifter Slips (Erie, USA) and placed in a sealed humidified hybridization chamber (Scienion, Germany) at 42°C for 48 h without shaking. After hybridization the array was washed and dried according to the Ambion protocol. The microarray data discussed in this study have been deposited in NCBI Gene Expression Omnibus (GEO, *http://www.ncbi.nlm.nih.gov/geo/*).

2.2.8 Data analysis and statistics for the pathogen array

Scanning of microarrays was performed with 5 µm resolution using a microarray laser scanner (Agilent). Features were extracted using the Agilent Technologies image analysis software (Version A7.5) using default settings for non-Agilent microarrays. Local background subtraction method was used and the background was adjusted globally to zero. Dye normalization was done using the rank consistency method and applying local weighted regression normalization (LOWESS). The ratio between both channels, the log ratio error and the p-values were calculated using default settings. Data analysis was carried out on the Rosetta Inpharmatics platform Resolver Buildt 4.2. Transcriptome analysis was carried out with two biological replicates for 36 h p.i. and at least three biological replicates for all other time points. Samples were derived from independent infections, RNA preparations, labeling reactions and hybridizations. Genes were selected using a 1.8-fold expression cut-off. All data of the individual sets comprising different time points was combined as Ratio Experiments using negative polarity for the color swap dye-reversal hybridizations. This was achieved by combining the Ratio Profiles - representing single hybridizations - using an error weighted average based on p-values.

Additionally, the microarray data were statistically analyzed using the significant analysis of microarray (SAM) algorithm V2.0 (www-stat.stanford.edu/_tibs/SAM/). SAM uses the standard deviation of repeated gene expression measurements to assign a score to each gene and estimates a false discovery rate (FDR) by permutations of the data. This analysis ascertains that genes identified as differentially expressed do not arise from a random

fluctuation of the large quantity of data generated. To identify genes whose expression differed significantly during the acute time course a one-class time course analysis with log2-data taken from Ratio Profiles was performed. For dye swap experiments fold change data were reversed, converted into ratio values and log2 transformed. A false discovery rate (FDR) of 0.46% and a delta value of Δ=0.49 for the cycle of development was applied. Additionally the FDR was calculated separately for each time point of the acute and persistent infection using a one-class analysis. Calculations are depicted as supplementary data. For the persistent infection a FDR of 1.5%, 18.6%, 1.3% and a delta value of Δ=0.73, Δ=0.17, Δ=0.327 for 24 h, 48 h and 72 h p.i. was applied and significant genes were combined for further analysis. The microarray data of SAM-positive genes were extracted using the Samster software (Mueller, A. et al., 2003) and Ratio Profiles – representing single hybridizations - or Ratio Experiments – representing all hybridizations derived from one time point – were clustered with Gene Cluster (Eisen, M. B. et al., 1998) using a self organizing map (SOM). Cluster calculations were displayed using TreeView (http://rana.lbl.gov/EisenSoftware.htm).

2.2.9 EB purification, RNA extraction labeling and hybridization

Purification was basically done as described by Mukhopadhyay (Mukhopadhyay, S. et al., 2004), with slight modifications. Briefly, 10^8 cells were lysed in 5 ml NP-40 (Fluka) complemented SPG [1% NP-40 per 1 x 10^7 cells] and were subjected to sonication for 5 min followed by a 20 min incubation step with DNaseI [1.6 µg/ml] (Grade II, Roche) on ice. Nuclei and bigger fragments were removed by a 5 min centrifugation step at 1000 x *g*. Post nuclear supernatant was layered on top of a 7 ml 50% - 25 ml 20% Ficoll-400 (Pharmacia) step gradient and was centrifuged at 18,500 x *g* for 1 h at 4°C in a swing out rotor. Interphase was collected and washed by addition of 10 ml SPG and centrifugation at 18,500 x *g* for 30 min. Pellet was resupended with a syringe and 5 min sonication to dissolve EB clumps and suspension was aliquoted and stored at minus 70°C for further use. Purity was assessed by electron microscopy on a Leo 906E Transmission Electron Microscope. RNA extraction, labeling and hybridization were performed as described before. The mRNA content was determined using a 4-fold over background cut-off following background subtraction.

2.2.10 Data analysis and statistics for the eukaryotic array

Scanning of microarrays was performed with 5 µm resolution using a microarray laser scanner (Agilent). Features were extracted with an image analysis tool Version A 6.1.1

(Agilent Technologies) using default settings. Local background subtraction method was used and the background was adjusted globally to zero. Dye normalization was done using the rank consistency method and applying local weighted regression normalization (LOWESS). Data analysis was carried out on the Rosetta Inpharmatics platform Resolver Built 4.0. Ratio profiles were generated from raw scan data by a processing pipeline which includes pre-processing (Feature Extraction) and post-processing (Rosetta Resolver) of data and error model adjustments to the raw scan data. Ratio profiles were combined in an error-weighted fashion (Rosetta Resolver) to create ratio experiments. Ratio experiments consisted of one or more ratio profiles. Expression patterns were identified using stringent analysis criteria of 1.8-fold expression cut-offs of the ratio experiments and an anti-correlation of the dye reversal ratio profiles. Anti-correlation was determined by using the 'compare function' to match two different hybridizations pairs and to decide how similar or dissimilar they were. In this way, only anti-correlated spots and vice versa were selected. By combining the first and the second criteria of analysis data points with low P-value (P-value < 0.01) were filtered out, making the analysis robust and reproducible. Additionally, by using this strategy the data selection was independent of error models implemented in the Rosetta Resolver system.

2.2.11 Real time PCR and normalization

Primers for quantitative real time PCR (qRT-PCR) were designed for selected genes using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and blasted against the human genome to get a high probability for *Cpn* specific products. Product specificity was controlled using the denaturing protocol of the ABI Prism 7000 program (Applied Biosystems). Standard curves were performed using total DNA from infected HEp-2 cells to measure the primer pair efficiency. qRT-PCR was performed in duplicates for at least three biological replicates. RNA was transcribed into cDNA using 2 µg total RNA, random nonamer primers (Amersham) and the Omniscript RT Kit (Qiagen) according to the manufacturers instructions. 20 µl of the derived cDNA was diluted with 500 µl H_2O and qRT-PCR was performed using 5 µl per well. Primer-pairs and SYBR-green mastermix were mixed with the cDNA according to the manufacturer's instruction (Applied Biosystems). SYBR-green uptake in double stranded DNA was measured on an ABI Prism 7000 thermocyler (Applied Biosystems). Ten putative housekeeping genes including 16S rRNA were picked using literature and the microarray data of this study to calculate a housekeeping index (HKI) for normalization purposes. The qRT-PCR experiments were performed for all time points and the accuracy of the chosen housekeeping candidates was

calculated with the geNorm program (http://medgen.ugent.be/~jvdesomp/genorm/). A set of the three most accurate housekeeping genes were combined to a housekeeping index (HKI) and used to normalize gene expression. Relative quantification was performed using the $2^{(-\Delta\Delta CP)}$ method including an efficiency correction (Pfaffl, M. W., 2001a) for the primers.

2.2.12. Microscopy

To analyze the establishment of the persistence for *C. pneumoniae* grown under iron-deficient conditions, infected host cells were fixed with paraformaldehyde and labeled with an anti-chlamydial-LPS antibody (Progen, Germany).

III RESULTS and DISCUSSION

3.1 Optimization of the infection, RNA extraction and labeling procedures

Performing microarray studies with obligate intracellular pathogenic bacteria is still hampered due to the following reasons. First, total RNA of cells infected with an intracellular pathogen consists of host and bacterial RNA with only a marginal part being that of bacterial origin. Second, in contrast to eukaryotic mRNA, bacterial mRNA does not possess a poly-A tail. Therefore the use of poly-T primers combined with specific promoters (like T7) as it is widely used for eukaryotic cDNA labeling is not applicable. So far, no technique exists that allows amplification of the bacterial mRNA out of the RNA pool. Attempts have been made to amplify bacterial mRNA using a genome directed primer set (Talaat, A. M. *et al.*, 2000b). However, it is still unclear if this procedure results in a linear amplification of the different RNA species and does not introduce a technical bias. Recently, several publications have tried different strategies to overcome these obstacles for *C. trachomatis* microarray analysis. A complete primer set of all open reading frames (ORF) for the *C. trachomatis* genome was constructed targeting only bacterial transcripts in the reverse transcription reaction (Belland, R. J. *et al.*, 2003b;Belland, R. J. *et al.*, 2003g). However, it has been shown that a complete primer set introduces a technical bias and the use of random primers is necessary to get reproducable results (Arfin, S. M. *et al.*, 2000b). Another group enriched the chlamydial RB by centrifugation prior to RNA extraction (Nicholson, T. L. *et al.*, 2003e). Bacteria sense their environment and mRNA expression changes occur within minutes (Brazas, M. D. *et al.*, 2005) which makes the enrichment of the chlamydial RB by centrifugation impractical. Therefore the improvement of the infection rate as well as RNA extraction and labeling methods is of great importance to perform microarray experiments with obligate intracellular bacteria.

3.1.1 Optimization of the infection procedure

It has been reported that *Cpn* infections with high Multiplicity of infection (MOI) can lead to cytotoxic effects (Al Younes, H. M. *et al.*, 2001b). Therfore, MOI from 0 up to MOI 40 were tested in order to optimize the amount of extracted bacterial RNA. Therefore, the integrity of the host cell layer was investigated for using high MOI *Cpn* infections. In addition, the ratio between eukaryotic and prokaryotic rRNA species was measured using the Agilent Bioanalyzer.

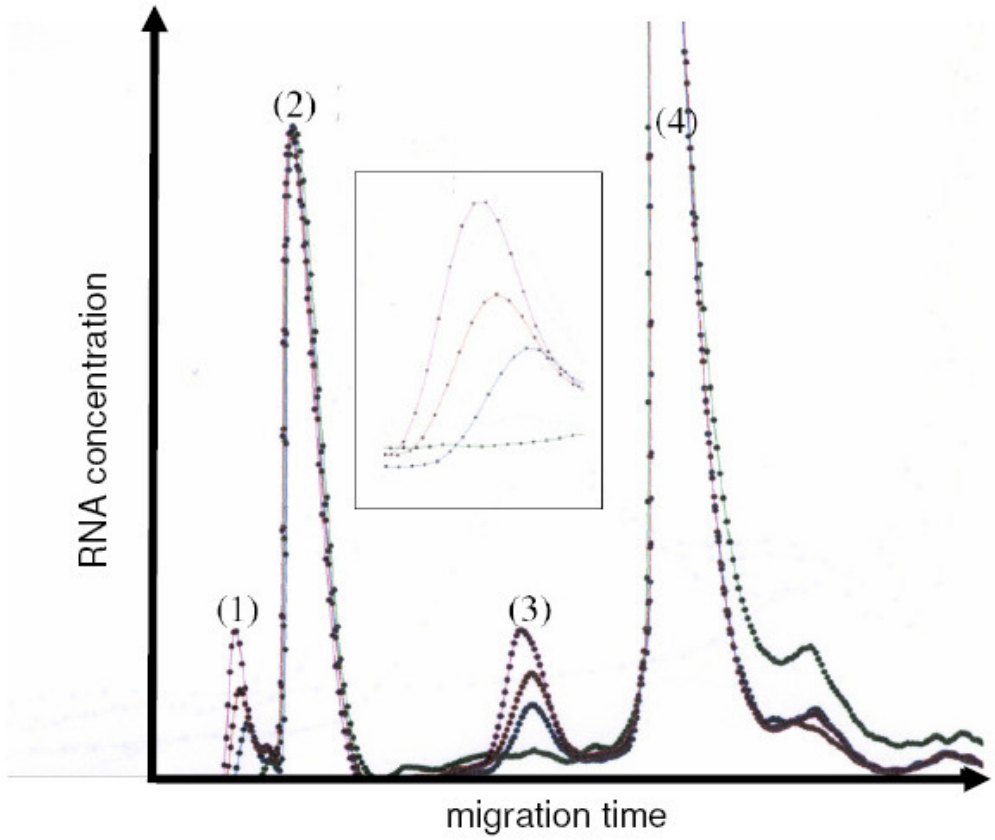


Figure 9 - **Determination of RNA concentration and purity using the Bioanalyzer RNA 6000** (Agilent Technologies). Visible are the peaks indicating the presence of chlamydial 16S rRNA (1) and 23S rRNA (3) as well as the eukaryotic 18S rRNA (2) and 28S rRNA (4) at 48 h p.i. HEp-2 epithelial cells were infected with *C. pneumoniae* using an MOI of 0, 5, 10, 15. The measurements were normalized using the 18S rRNA peak to make the samples comparable. The amount of chlamydial RNA increased reflecting higher MOI used. (Magnification of peak (3) is shown in the square). X-axis represents the RNA migration time and the Y-axis presents the amount of RNA. All measurements were normalized to eukaryotic 18S rRNA.

The use of MOI 30 and 40 resulted in an increased detachment of host cells and led to disturbance of the cell layer integrity at time points from 48 h to 72 h p.i. Consequently, an MOI of 30 was used only for early time points (6 h and 12 h p.i.) as detachment of the host cells has not been noticed for these time points. For later time points HEp-2 cells were infected with an MOI of 0, 5, 10, and 15. The RNA was extracted with the standard TRIzol protocol recommended by the supplier and measured with the Bioanalyzer RNA 6000 (Agilent Technologies). Higher MOI resulted in an increased bacteria versus host rRNA ratio (Figure 9). Consequently, for later time points from 18 h p.i. to 72 h p.i. an MOI of 15 was used.

3.1.2 Optimization of the RNA extraction procedure

The extraction of mRNA extraction is a crucial step for transcriptome and gene expression analysis targeting obligate intracellular bacteria. It is challenging since the vast majority of total RNA is of host cell origin. Recently a method for enrichment of bacterial mRNA extraction out of an RNA pool has been published (Microbeenrich, Ambion). It is based on removing the eukaryotic RNA with the use of polyT primers that bind to the eukaryotic polyA tail. This method is time and resource consuming and also includes the possibility of introducing a bias into the experiments as Chlamydiae have a high AT rich genome. Thus, when working with obligate intracellular organisms, improvement of extraction methods is of great importance. In this study the manufacturer's instructions for RNA isolation with TRIzol was modified by introducing a sonication and RNA precipitation step with glycogen. 23S and 28S rRNA peaks were measured with the Agilent Bioanalyzer and the ratio between these peaks served as an indicator for the prokaryotic and eukaryotic fraction of the total RNA. Using sonication during the usage of TRIzol® larger quantities of bacterial 16S rRNA and 28S rRNA were achieved compared to the untreated samples (Figure 10). The use of sonication led to an increase of bacterial RNA from 11% to 22% of the total amount of extracted RNA. Additionally, improved recovery of chlamydial RNA was achieved when glycogen was added during precipitation. Figure 10 illustrates the increased yield in bacterial RNA when glycogen was included in the precipitation procedure, as compared to the untreated control. The use of glycogen led to an enrichment of bacterial RNA to about 29% of the total amount of extracted RNA. It was speculated that the lysis of the bacterial cell was not sufficient without the sonication process. Additionally, the use of glycogen as an RNA carrier for precipitation increased the percentage of bacterial RNA in the extraction procedure. It was speculated that glycogen might lead to an increased precipitation for small RNA fragments and therefore favors bacterial RNA. Taken together, using the combination of sonication for lysis and the use of glycogen as an RNA carrier achieved a high recovery rate for bacterial RNA.

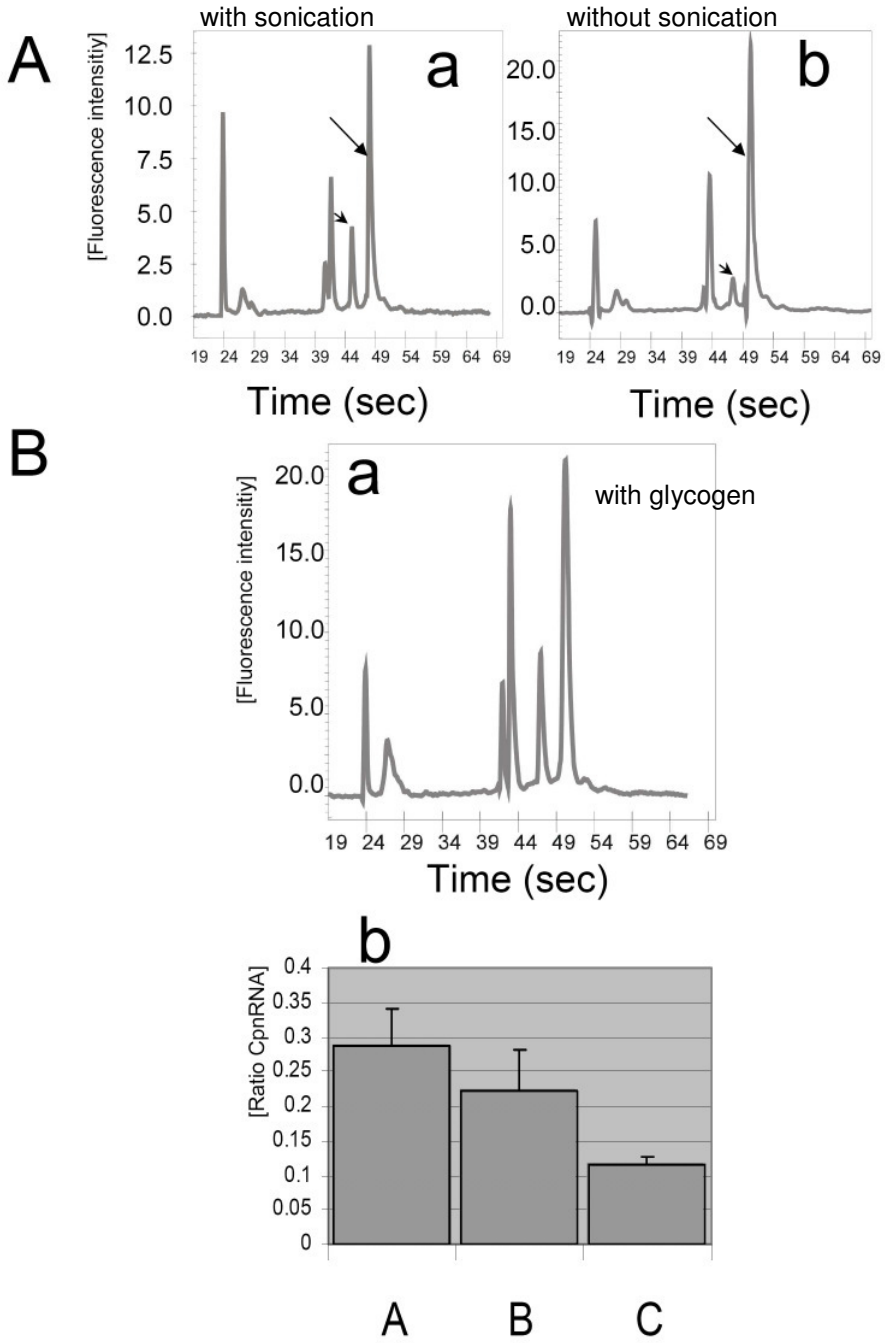


Figure 10 - **RNA Isolation with and without the use of sonication.** (A) *C. pneumoniae* infected HEp-2 cells (MOI 15) were lysed at 2 d p.i. with the described TRIzol protocol without the use of glycogen and (a) with 5 min sonication or (b) without a sonication step. Sonication leads to an increased recovery of chlamydial RNA. Electropherogram obtained with the Agilent Bioanalyzer 2100 software shows 16S, 18S, 23S (arrowhead) and 28S (arrow) rRNA. (B) RNA isolation with the use of glycogen. (a) *C. pneumoniae* infected HEp-2 cells (MOI 15) were lysed 2 d p.i. with the described TRIzol protocol and the use of sonication and glycogen. Electropherogram obtained with the Agilent Bioanalyzer 2100 software. (b) Use of glycogen and sonication leads to increased recovery of chlamydial RNA which was measured by calculating the ratio between 23S and 28S rRNA. A – use of glycogen and sonication, B – use of sonication alone, C – standard TRIzol protocol.

3.1.3 Labeling and hybridization

So far, as for eukaryotic RNA, no standard method for labeling and hybridization exists for intracellular bacteria. Recently in a transcriptome study done with *C. trachomatis* a 3' ORF specific primer set covering all genes was used (Belland, R. J. *et al.*, 2003f). However, it has been shown that random priming is required for accurate measurement of gene expression levels in bacteria (Arfin, S. M. *et al.*, 2000a). It was discussed that the use of a 3` ORF primer set leads to a bias because of unequal hybridization efficiencies and widely differing degradation rates for the 3' ORF regions. Therefore, random nonamers were used to convert the RNA to cDNA and to label the cDNA. To minimize dye specific effects for the performed dye swap experiments an indirect aminoallyl labeling was performed to which in a second step a NHS-ester-base was coupled to the cDNA. This two-step-labeling led to increased signal intensity and also reduced the bias between Cy3 and Cy5 dye swap experiments compared to conventional labeling protocols. The modifications in the infection procedure, RNA extraction and labeling protocols led to signal intensities that allowed performing microarray experiments for the acute and iron persistent infection.

3.1.4 Development and validation of the *C. pneumoniae* specific microarray

For performing transcriptome studies with obligate intracellular bacteria the development of the oligomicroarray to be used needs particular precaution. Total RNA consists of host and pathogen RNA, with the latter representing only a marginal part. To avoid cross-hybridizations as a result of the abundant host RNA the oligomicroarray has to be specific for *C. pneumoniae*. The development of the oligomicroarray was done in cooperation with MWG AG (Ebersberg, Germany). 50mer oligos for each ORF of the *C. pneumoniae* strains *CWL029*, *AR39* and *J138* were designed. Each gene of the three strains is targeted by one 50mer oligo. Genes with homologous sequences are represented only by one oligo on the array. The oligos were blasted against the human genome using the NCBI database in order to minimize host RNA cross-hybridization effects. Furthermore, cross-hybridization was tested experimentally. Human RNA was extracted from uninfected HEp-2 cells, labeled and hybridized exactly as done for infected cells. Oligo spots showing cross-hybridization were redesigned and the verification was repeated. Figure 11 illustrates the final version of the oligoarray with the vast majority of oligos showing acceptable low or no cross-hybridization.

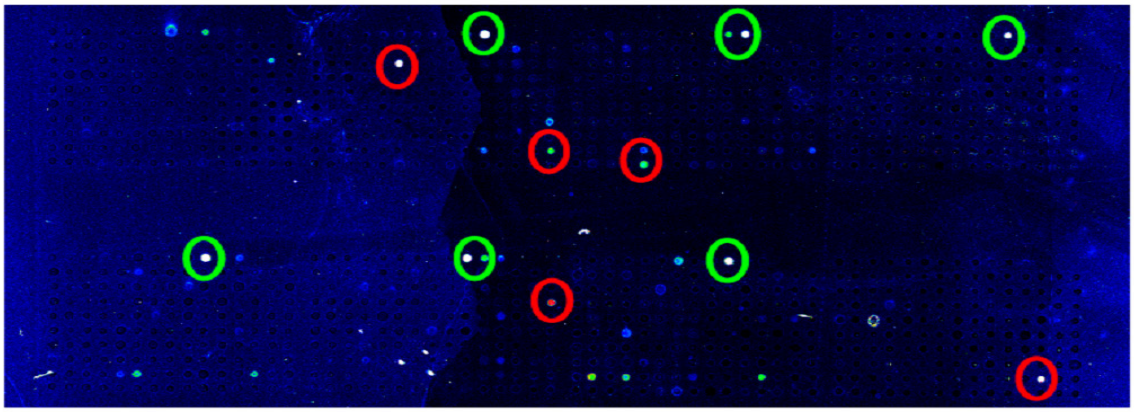


Figure 11 - **Hybridization of eukaryotic RNA to the *C. pneumonaie* specific array.** The same extraction, labelling method and hybridization conditions had been used as for the infected cells. Only little or no cross-hybridization occurred. White dots show a high level, red dots or green dots medium and blue dots or black dots a low level of cross hybridization. Human control oligos are marked with a green and chlamydial oligos with a high crosshybridization with a red circle.

3.1.5 Discussion

In the recent years transcriptome studies became more and more widely used to study pathogen – host cell interactions from a global point of view. While for eukaryotic cells several methods have been established (e.g. Affimetrix, Agilent, T7 based methods) analyzing intracellular organisms is still far from being standardized. This requires special precaution for performing these experiments. Recently, attempts have been made to amplify bacterial RNA (Talaat, A. M. *et al.*, 2000a;Belland, R. J. *et al.*, 2003e). However, a precondition for the amplification of RNA populations is a linear amplification procedure. This is hampered due to the reason that bacterial mRNA does not posses a polyA tail. Therefore it has not yet been verified that amplification of bacterial RNA is non-biased. Moreover, enrichment of bacteria is still very common (Nicholson, T. L. *et al.*, 2003d). However, environmental stress leads to minute changes in the mRNA population and therefore to false results. The establishment of the RNA extraction, the labeling and hybridization methods as well as the *Cpn* specific array are therefore crucial steps. The improvement of the RNA extraction for *Cpn* methods resulted in an about 3 x fold increased yield of pathogen RNA. So far it is the first time a pathogen-specific oligomicroarray has been designed using a blast comparison to the human genome. This strategy resulted in a very low cross-hybridization. Take together, these methods open up the possibility to study the *Cpn* transcriptome in the acute and persistent infection and represent a major improvement compared to earlier microarray studies done with *Ctr.*

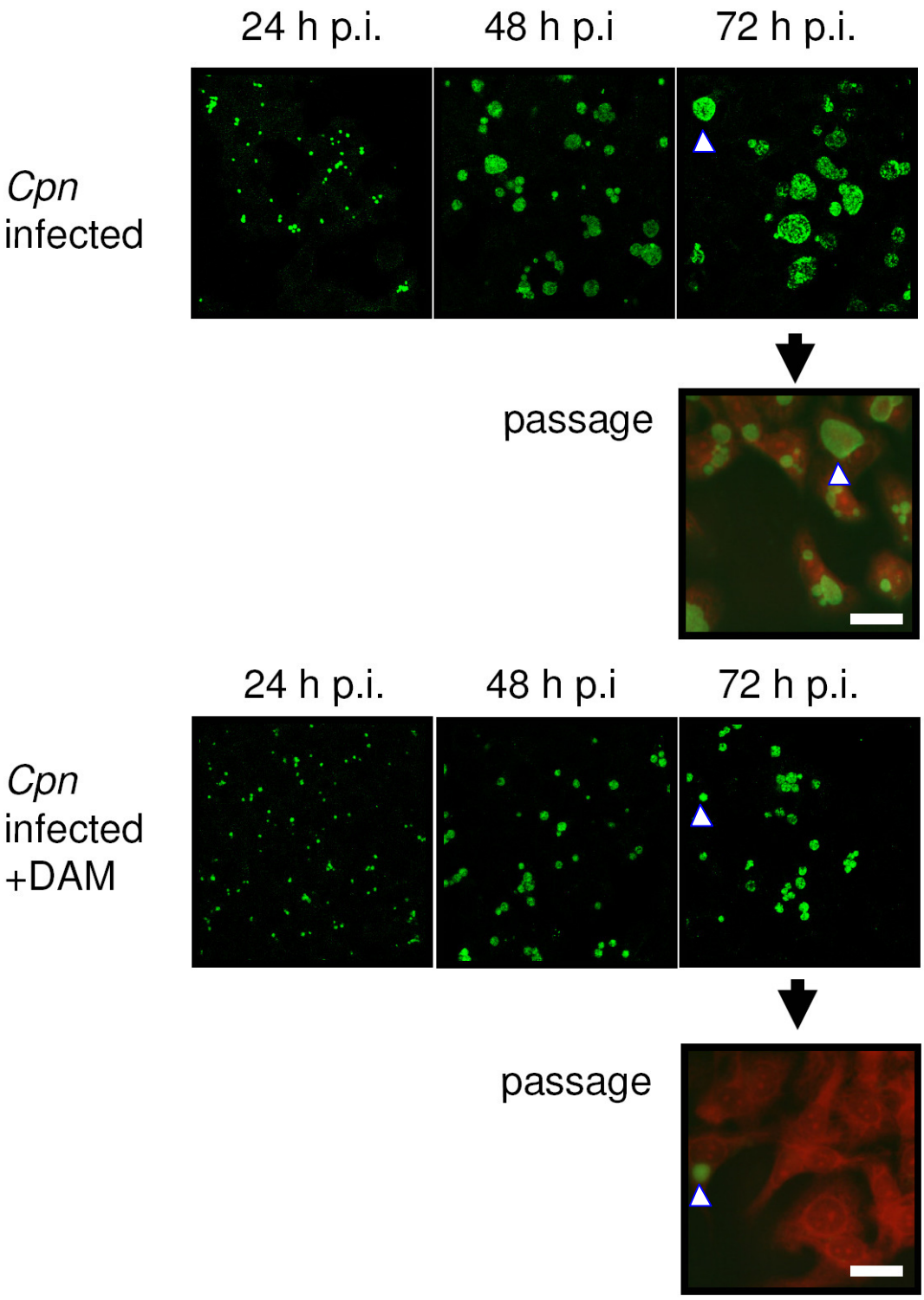


Figure 12 - **Fluorescence pictures of the acute and the DAM mediated iron-depletion infections.** HEp-2 cells were infected with *C. pneumoniae* (MOI 5) for the acute infection and additionally incubated with DAM to obtain a persistent infection. The infected cells were lysed 72 h p.i. and HEp-2 cells infected with a 1:250 dilution of the lysate to determine the progeny of the acute and persistent infection. The bar indicates 10 µm. Labeling has been done with with an α-LPS antibody (Daco). The arrowheads point to typical chlamydial inclusions.

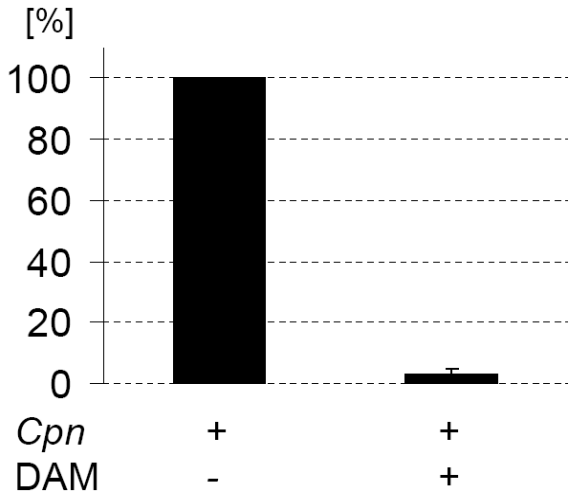


Figure 13 - **Quantitative determination of the progeny of the acute and persistent infection.** Cells were infected with C. pneumoniae (MOI5) for the acute infection and additionally incubated with DAM to obtain a persistent infection. The infected cells were lysed after 72 h p.i. and HEp-2 cells infected with a 1:250 dilution of the lysate to determine the progeny of the acute and persistent infection. Compared to the acute infection the persistent infection led to a drastically reduced progeny of 3.3%. The error bars display the standard deviation of five independent counts.

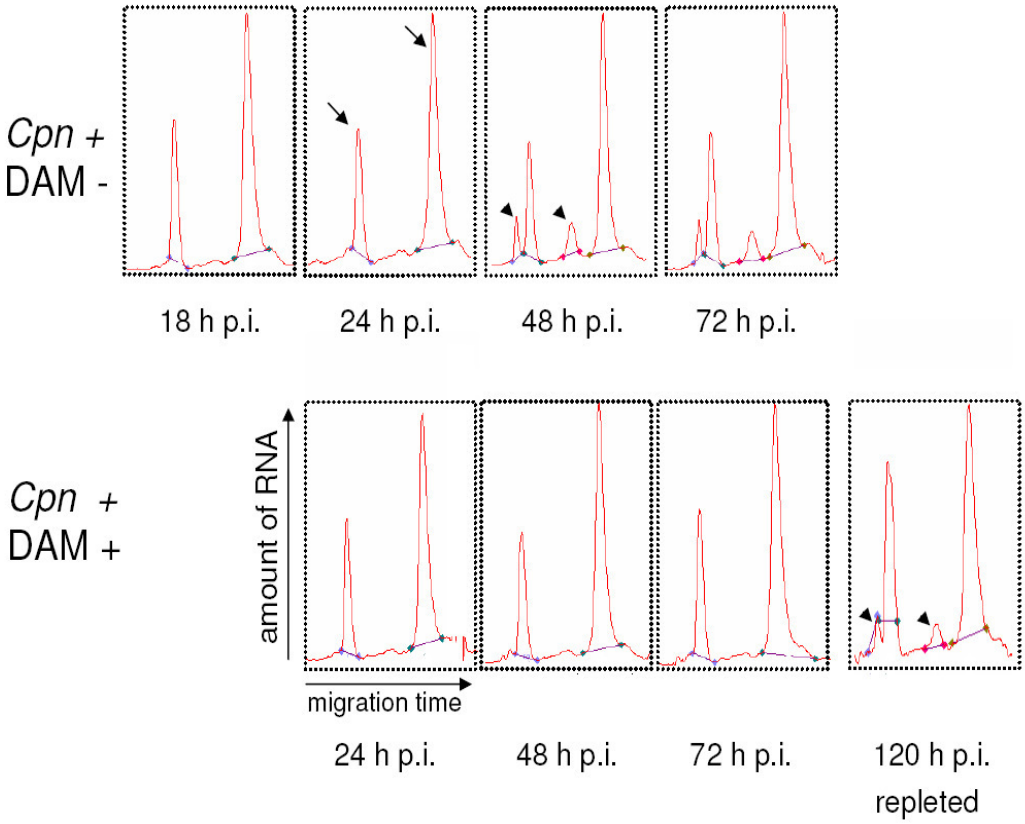


Figure 14 - **Bioanalyzer 2100 profiles of the acute and the iron depletion infections**. Electropherogram was obtained with the Agilent Bioanalyzer 2100 software. *C. pneumoniae* infected Hep-2 cells were lysed at the indicated time points with the described optimized TRIzol protocol. The sample for 120 h p.i. was repleted (DAM-containing medium replaced with normal medium) at 72 h p.i. Peaks indicate the amount of bacterial (arrowheads: 16S rRNA and 23S rRNA) and host rRNA (arrows: 18S rRNA and 28S rRNA)in the RNA pool. Compared to the host cell rRNA the amount of bacterial rRNA increases throughout the developmental cycle. In the DAM mediated iron-depletion persistence only little peaks can be seen. After repletion of the persistent infection 16S rRNA and 23S rRNA were increased indicating the resumption of the developmental cycle.

3.2 Transcriptional changes during the *C. pneumoniae* developmental cycle

RNA samples from infected cells were taken from 6 h to 72 h post infection to measure the *Cpn* transcriptome throughout the *Cpn* developmental cycle. The acute infection was verified using microscopy as well as measurement of the RNA samples with the Bioanalyzer 2100 (Figure 12, Figure 13, Figure 14). Transcriptional changes were defined by comparison of expression levels to 48 h p.i. as a mid-cycle reference time point. Statistical analysis with Significance Analysis of Microarrays (SAM) software (Tusher, Tibshirani, and Chu, 2001) showed the lowest false discovery rate (FDR) for mid cycle time points. This indicates that the variation of expression was lowest at these time points and that therefore 48 h p.i. was suitable as a reference time point. The experimental set-up is consistent to a recent publication for *C. trachomatis* (Nicholson et al., 2003). By choosing a 1.8-fold expression cut-off, 651 *Cpn* genes were determined as differentially expressed for at least one time point during the cycle (61% of 1062 *Cpn* features), whereas the remaining genes did not meet this criterion.

3.2.1 The *C. pneumoniae* transcriptome can be grouped into 12 clusters of expression and four cluster classes

The statistical analysis of the time course using SAM software identified 754 out of 1062 *Cpn* features as significantly expressed (Figure 15) with 533 genes meeting the 1.8-fold cut-off criterion (Figure 16)(Supplementary-Table 1). The expression of individual genes throughout the *Cpn CWL029* developmental cycle was compared to expression at a midpoint in the developmental cycle to generate expression profiles. Based on these profiles, genes with similar expression changes formed 12 clusters using the self-organizing map algorithm (SOM). The *Cpn* clusters were grouped into the 'Early', 'Mid' and 'Late' classes as described for *Ctr* (Belland, R. J. *et al.*, 2003d;Nicholson, T. L. *et al.*, 2003c). Existing expression data for Chlamydiae mainly define the time point in the cycle at which new transcription begins. Here, in contrast the gene expression profiles were used for categorization. This turned out to be more appropriate for comparing the time specific relevance of a certain cluster of genes in the cycle to their proposed functions. Additionally, a new gene expression class containing genes with steadily increasing expression at the end of the developmental cycle was defined and termed 'Tardy' class. As bacteria show an immediate correlation between mRNA transcription and protein synthesis it can be presumed that transcriptional changes are immediately linked to temporal processes throughout the cycle of development.

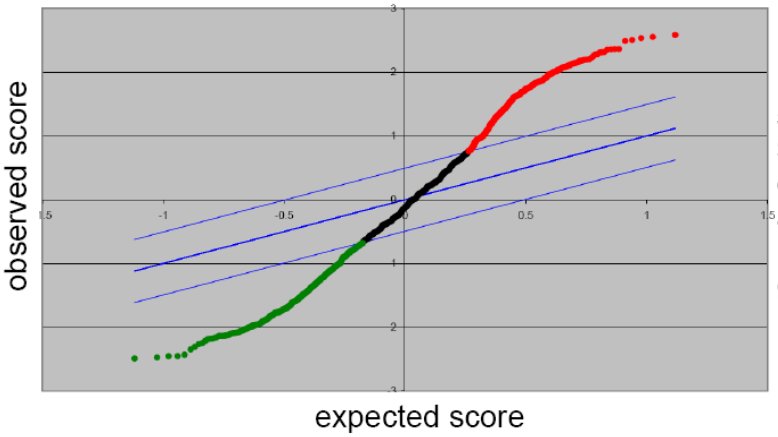


Figure 15 - **One class significance calculation done with SAM software for the acute time course.** 754 genes were calculated as significantly regulated. Down-regulated genes are indicated in green, up-regulated in red.

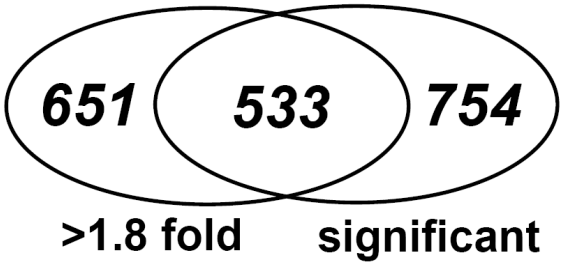


Figure 16 - **Significantly regulated genes during acute infection.** 754 genes were calculated as significantly expressed using SAM software. Out these 533 genes were regulated more than 1.8-fold at least at one time point throughout the cycle.

3.2.1.1 The 'Early' gene cluster class

Genes with the highest transcription levels at 6 h p.i. were defined to belong to the 'Early' cluster class (cluster 1 and 2; Figure 17). This included cluster 1 and 2. Following 6 h p.i. both clusters showed declining expression profiles. Cluster 1 reached its minimum expression level at 12 h post infection. Genes of cluster 2 revealed a slow-going decrease and reached their minimum expression levels between 24 h and 36 h post infection. None of the clusters showed significant up-regulation at later time points. The expression profiles of cluster 1 suggest a function very early in the cycle, whereas genes belonging to cluster 2 play a part in events up to the middle of the cycle. The ‘Early’ cluster class comprises a number of prominent genes, including *euo* (Zhang, L. *et al.*, 1998;Kaul, R. *et al.*, 1997;Zhang, L. *et al.*, 2000), considered to be a classical early gene, a minor proportion of the polymorphic membrane proteins (*pmp5.2*; *pmp12*; *pmp17.3*) (Grimwood, J. *et al.*, 2001a) and genes coding for glycolysis (*pgk* (*Cpn0679*), *dhnA*), transport (*oppA2*; *oppA3*; *oppA4*; *oppD*) and phospholipid metabolism (*htrb*). Genes coding for proteins involved in DNA metabolism (*recF*, *recC*, *nth*, *xerC*) and ribosomal proteins (*rl34*, *rl7*, *rs21*, *rl31*), as well as the glycerophospholipid metabolism genes *plsB* (*CPn0958*), *plsC* (*CPn0569*), *pgsA1* (*CPn0615*), *pssA* (*CPn0983*) and *psdD* (*CPn0839*), resulting in the synthesis of the essential membrane components phosphatidylglycerophosphate and phosphatidylethanolamine are also members of the 'Early' class. Initial stages of *Cpn* infection therefore involve induction of energy metabolism and transport proteins, as well as *de novo* synthesis of cellular components such as phospholipids.

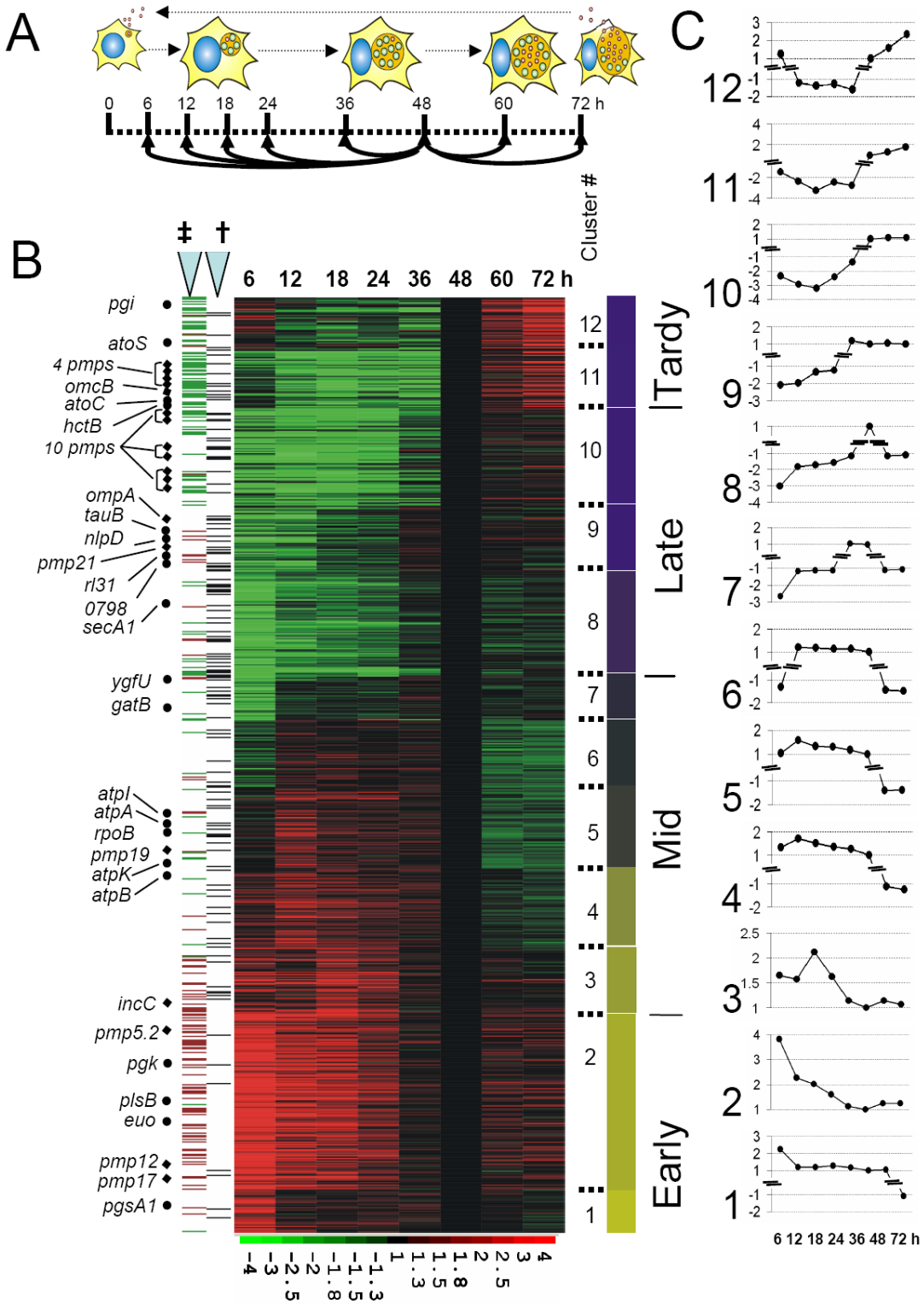


Figure 17 – ***Cpn* transcriptome in the acute infection.** (A) Cycle of development, timescale and experimental design. Cells were infected at time point 0, RNA was extracted at the indicated time points, labeled and hybridized with 48 h p.i. as a common reference. (B) SOM cluster (YDim = 12) of 754 significantly expressed genes during the cycle of development. Bars on the left indicate: (‡) the direction of expression of 207 significantly regulated genes with an expression >+/- 1.8-fold in the persistent infection with green being down- and red up-regulated and (†) 127 genes coding for proteins present in the EB (Vandahl, B. B. *et al.*, 2001a). Cluster number and cluster groups are represented on the right. Genes mentioned in the text are depicted. Gene expression is displayed in fold change represented by the color bar beneath the figure. Significant features were determined using Ratio Profiles with SAM and SAMSTER, Ratio-Experiments were clustered with GeneCluster software using a one class time course analysis and plotted with Treeview software. (C) Cluster profiles of the cluster represented in B. Gene identification numbers, gene definitions and gene names can be consulted at **http://www.stdgen.lanl.gov**. and in Supplementary-Table 1 .

3.2.1.2 The 'Mid' gene cluster class

Genes with increasing expression at the beginning of the cycle and decreasing expression at the end of the cycle were defined as ‘Mid’ class genes. Genes belonging to this class had an expression peak in the middle of the cycle. Clusters 3 to 7 were grouped to the 'Mid' cluster class. Cluster 3 expression peaked at 18 h p.i., whereas cluster 4 and 5 expression peaked at 12 h post infection. This suggests that genes of these clusters take part in time specific events during the cycle of development. Prominent genes of the 'Mid' cluster class are those involved in transcription and translation, genes of the ATP-synthase (*atpA*, *atpB*, *atpK*, *atpI*) and the synthesis of cofactors like biotin and folate. The type three secretion chaperone *lcrH2*, *ftsK* involved in cell division, and *incC* coding for an inclusion protein also fit this profile. It can be assumed that transcription of genes of the 'Mid' cluster class paves the way for a changed energy metabolism, the beginning of *Cpn* replication and cell division, as well as the growth of the chlamydial inclusion.

3.2.1.3 The 'Late' gene cluster class

The 'Late' gene cluster class includes clusters 8 to 10. Genes of these clusters were switched on at 36 h p.i. or even later during the cycle of development and showed a constant expression profile until 72 h p.i. of the acute developmental cycle. Examples of classic late genes in the literature are *hctA* (Hackstadt, T. et al., 1991) as well as *parB* and *ftsH*, necessary for binary fission (Gerard, H. C. *et al.*, 2001b). All of these genes belong to the ‘Late’ cluster class. Noteworthy is the accumulation of several genes coding for polymorphic membrane proteins (pmps) in the ‘Late’ cluster class (Figure 17).

3.2.1.4 The ‘Tardy’ gene cluster class

Additionally to the described classes, a new gene expression class containing genes with steadily increasing expression at the end of the developmental cycle was defined. This class was termed as the 'Tardy' gene cluster class and is different from the 'Late' cluster class that showed a constant expression profile at late time points. The ‘Tardy’ cluster class comprises clusters 10 and 11. Further, the ‘Tardy’ gene class showed a difference to the ‘Late’ class when comparing the transcriptome data to a proteomics study characterizing the EB protein content (Vandahl, B. B. *et al.*, 2001a) and to the EB mRNA content. Both are discussed in detail in the next two chapters.

3.2.2 Genes coding for proteins present in the elementary bodies are significantly linked to late but not tardy gene clusters

It has been discussed that genes expressed at late time points might play a role in events specific for the end of the developmental cycle, especially for RB to EB re-differentiation (Shaw, E. I. *et al.*, 2000a). The transcriptome data of the acute developmental cycle were compared to a proteomic study targeting the protein composition of chlamydial elementary bodies (EB) (Vandahl, B. B. *et al.*, 2001c). Out of the 754 significantly regulated genes 127 matched with the 165 published EB proteins (Figure 17). Using LACK (Kim, C. C. et al., 2003) 28 genes linked to cluster 8 ($n \geq 28$, $p < 0.01$) and 15 genes linked to cluster 9 ($n \geq 15$, $p < 0.05$) were determined. For clusters 10 and 11, 16 genes ($n \geq 16$, $p = 0.27$) and 10 genes ($n \geq 10$, $p = 0.28$) were linked to EB specific proteins, whereas only 4 genes were found for cluster 12 ($n \geq 4$, $p = 0.94$). Cluster 11 includes *omcB*, a major component of the EB. 13 polymorphic membrane proteins were found in clusters 10 and 11. The majority of genes of the EB proteins were significantly connected to clusters 8 and 9, while cluster 10 of the 'Late' cluster class and cluster 11 and 12 of the 'Tardy' cluster class contributed only with a minority of genes. Genes belonging to cluster 12 revealed an increased expression at 6 h p.i. and also at 60 h to 72 h post infection. Between 12 h p.i. and 48 h p.i. cluster 12 showed decreased expression. Moreover, genes of cluster 12 were not linked when comparing to the described EB proteins study (Vandahl, B. B. *et al.*, 2001d). This is an astonishing finding as it was expected that genes expressed at last in the cycle contribute most to the EB protein composition. Recently it was shown that RNA carry-over from late stages of the infection cycle into EB is possible (Douglas, A. L. *et al.*, 2000e). This provided evidence that initial translation in the cycle might be directed from stable transcripts present in the infected EB form. So far no evidence exists that suggests that carry-over message is functional at early time points. It therefore remains open to discussion whether carry-over of pre-synthesized mRNA allows a ‘hot start’ of translation right after infection, and further analysis will be needed to clarify this issue. Consequently, the use of the 'Tardy' gene cluster class might be useful for future consideration as it emphasizes the differences to the 'Late' gene cluster class.

3.2.3 The mRNA profile of the elementary bodies is connected to the 'Tardy' cluster class

To investigate the mRNA content of *Cpn* elementary bodies, EB were purified as described. Purity of the EB preparation was verified by electron microscopy before and after the purification (Supplementary-Figure 1). Subsequently, mRNA was extracted, labeled and hybridized as described. Two self–self hybridizations were performed, and a cut-off of 900 intensities of the processed signals was used to determine the EB specific mRNA content. 47 mRNAs were detected with this cut-off (Supplementary-Table 2). Using LACK software the EB specific mRNAs were compared to the cluster class classification. Only the Tardy cluster class was significantly linked ($n \geq 11$, $p=0.02$; Table 2) to EB mRNAs. For Late ($n \geq 14$, $p=0.56$), 'Mid' ($n \geq 5$, $p=0.99$) and 'Early' ($n \geq 13$, $p=0.21$) cluster classes no significant linkage was observed. This finding strengthens the validity of the novel category of tardy genes, as the smaller 'Tardy' cluster class has a greater impact on the EB mRNA content compared to the larger 'Late' cluster class.

Table 2 - **Comparison of EB associated gene-products with the cluster calculation of the developmental cycle.** The prevalence of 127 EB associated gene-products (Vandahl, B. B. *et al.*, 2001b) in the 12 clusters of the acute infection was calculated using the LACK software. For cluster 8 and 9 a significant correlation with EB associated gene-products was found, whereas clusters 5, 7, 10 and 11 did only contribute marginally but not significantly.

Cluster #	Cluster Size	Hits	Data Size	Full Dataset	p-Value
12	42	4	754	127	(p>=4)=0.94
11	48	10	754	127	(p>=10)=0.28
10	80	16	754	127	(p>=16)=0.27
9	50	15	754	127	(p>=15)=0.01
8	86	28	754	127	(p>=28)<0.01
7	34	8	754	127	(p>=8)=0.20
6	55	8	754	127	(p>=8)=0.73
5	65	13	754	127	(p>=13)=0.29
4	64	10	754	127	(p>=10)=0.65
3	53	7	754	127	(p>=7)=0.81
2	142	5	754	127	(p>=5)=0.99
1	34	2	754	127	(p>=2)=0.99

3.2.4 Differential gene expression accounts for changing requirements throughout the cycle of development

During the cycle of development, *Cpn* undergoes morphological changes reflected by expression changes of groups of functionally related genes. Therefore, the developmental cycle was analyzed in more detail on the basis of selected functional categories (gene ontology categories) and sub-categories (*http://www.stdgen.lanl.gov*) (Figure 18). A schematic overview is presented in Figure 19.

3.2.4.1 Cell envelope and cell division

The *Cpn* genome encodes a family of 21 polymorphic membrane proteins (pmp), which are predicted to be localized in the bacterial outer membrane (Kalman, S. *et al.*, 1999e). Only *pmp5.2* (*Cpn0019*), *pmp12* (*Cpn0452*) and *pmp17.3* (*Cpn0470*) showed an early up-regulation at 6 h p.i., whereas *pmp19* (*Cpn0539*) had a mid expression profile. However, the majority is not switched on before 48 h p.i., showing an accumulation in clusters 10 and 11, most likely reflecting their importance in the process of RB to EB re-differentiation. *Pmp19* showed an unique expression profile in the family of the pmps. It was up-regulated at 12 h p.i. with a decreasing expression profile until the end of the cycle. *Pmp19* has been shown to be a major antigen of *Cpn* (Mueller et al., 2004). *Pmp21*, *pmp6* and *pmp20* code for described autotransporters (Vandahl, B. B. et al., 2002;Wehrl, W. et al., 2004a), are expressed late in the cycle. *IncB* and *incC*, both associated with the chlamydial inclusion (Fields, K. A. *et al.*, 2002), showed a peak of expression between 12 h p.i. and 18 h post infection. Classical markers for RB to EB differentiation are *ompA* and *omcB* (Shaw, E. I. *et al.*, 2000b). While *ompA* is a member of the 'Late' cluster class, *omcB* has been grouped to the 'Tardy' cluster class. The expression of peptidoglycan genes showed a time specific expression, with *murE* in the 'Early' cluster class and *murA*, *murC*, *murD* and *murF* in the 'Late' cluster class. Of the cell division genes present in *E. coli,* Chlamydiae possess only *ftsY* and *ftsW*, but not *ftsZ* (Rothfield, L. et al., 1999;Bramhill, D., 1997). Therefore, a role for peptidoglycans has been discussed not only in the transition from RB to EB prior to their release, but also in RB division (Chopra, I. *et al.*, 1998b). Both *ftsY* and *ftsW* had highest expression in the mid cycle. Further, the genome also encodes for genes not essential for cell division, such as *parB*, a gene of the 'Late' class, and *rodA*, associated with the bacterial shape. The latter showed an expression with two peaks, suggesting a role in RB and in EB synthesis. The essential inner membrane-bound protease *ftsH* belongs to the 'Late' cluster class suggesting a role mainly in RB to EB conversion.

3.2.4.2 Secretion processes

The putative chlamydial Type Three Secretion System (TTSS) (Stephens, R. S. *et al.*, 1998d) is thought to play a key role in host-pathogen interactions by channeling different effectors into the host cell (Shirai, M. *et al.*, 2000;Kalman, S. *et al.*, 1999d;Read, T. D. *et al.*, 2000b). Determinants belonging to the TTSS are located in five genomic islands that showed different expression profiles corresponding from 'Mid' and 'Late' cluster classes (Figure 18). The data are highly consistent with a recent report (Slepenkin, A. *et al.*, 2003b). Noteworthy, the two chaperones *lcrH1* and *lcrH2* showed differential expression patterns, with the former being grouped in the 'Tardy' class whereas the latter had an expression maximum at 18 h p.i. with down-regulation towards the end of the cycle and was therefore grouped in the ‘Mid’ class. In addition, *Cpn* possesses eight genes coding for the Type Two Secretion System (TIISS) of which most had a peak expression between 6 h and 12 h p.i. and were therefore grouped in the ‘Mid’ cluster class. This indicates different roles of the secretion systems throughout the developmental cycle. Not many secreted proteins are known so far. One of the first discovered for *Cpn* is CPAF (chlamydia proteasome-like activity factor) (*Cpn1016*), which degrades host transcription factors (Heuer, D. *et al.*, 2003b). This was a 'Late' class gene showing constant expression until the end of the cycle. Recently, a screen was published leading to the prediction of 24 putative genes coding for TTSS proteins in the *Cpn* genome (Subtil, A. *et al.*, 2005b). Gene expression for these TTSS candidates can be divided into early, mid and tardy expression profiles (Figure 20). Interestingly, the expression profile of the mid group correlates with the expression profile of the TTSS chaperone *lcrH2*, whereas for the tardy group the expression profiles correlate to the one observed for *lcrH1*. This indicates different time specific functions for these genes at early, mid and tardy stages of the cycle.

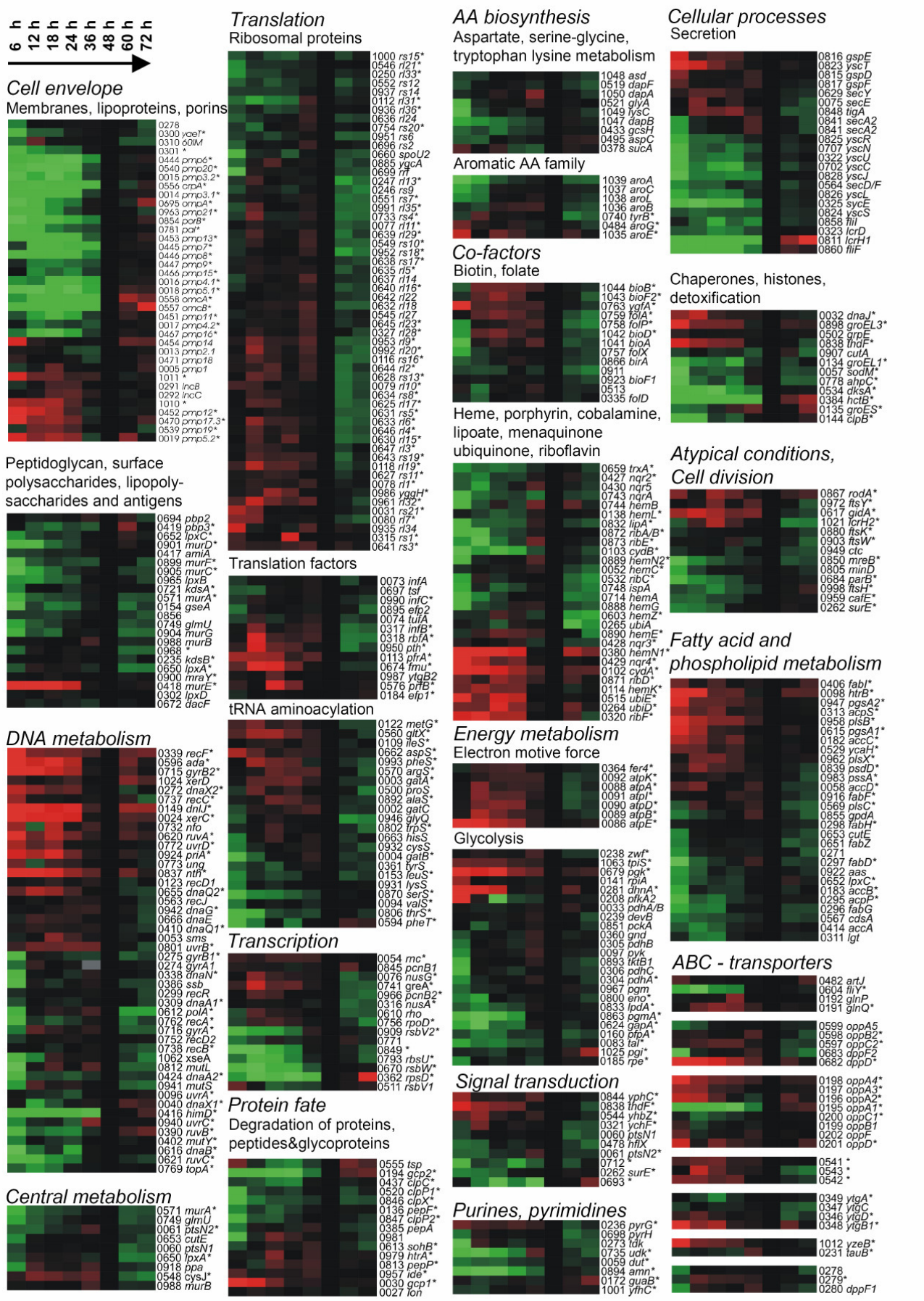


Figure 18 - **Functional grouping and cluster analysis.** Detailed expression profiles of functionally related sets of genes according to Los Alamos Database. Clusters were created using SOM cluster analysis using the color scale as described for Figure 17 . Genes that were calculated as significantly regulated with SAM 2.0 are indicated with an asterix. Gene identification numbers, gene definitions and gene names can be consulted at http://www.stdgen.lanl.gov.

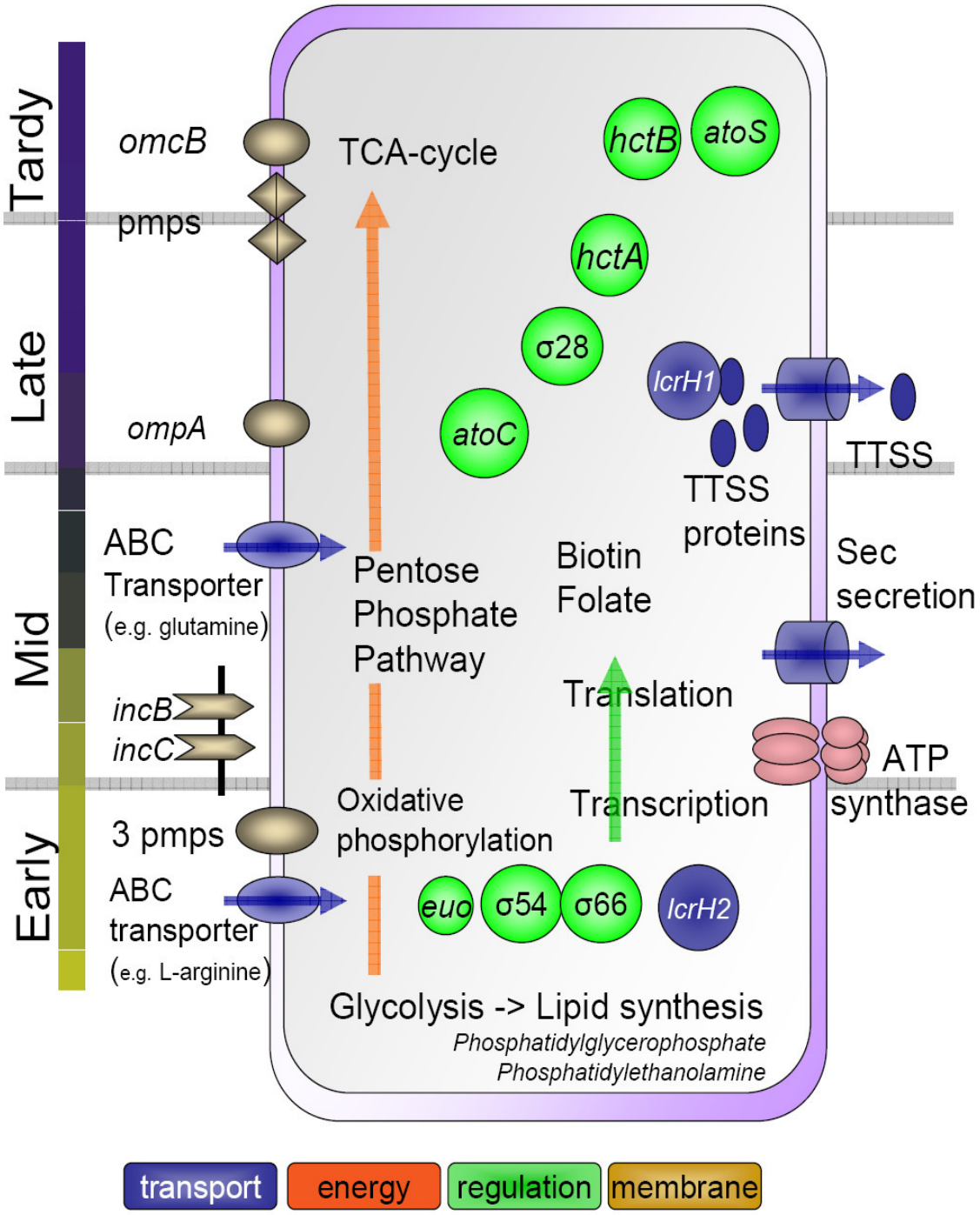


Figure 19 - **Schematic overview of important cellular functions according to the time of expression.** The illustration presents an overview of a *C. pneumoniae* cell and the time point of appearance of expression of genes involved in important cellular functions. Illustreated are the four cluster classes, the 12 clusters and genes involved in transport functions (blue), energy metabolism (red), transcriptional or translational regulation (green) and genes coding for membrane proteins (brown). The position of the genes is reminiscent to the grouping in the cluster profile. Gene identification numbers, gene definitions and gene names can be consulted at http://www.stdgen.lanl.gov.

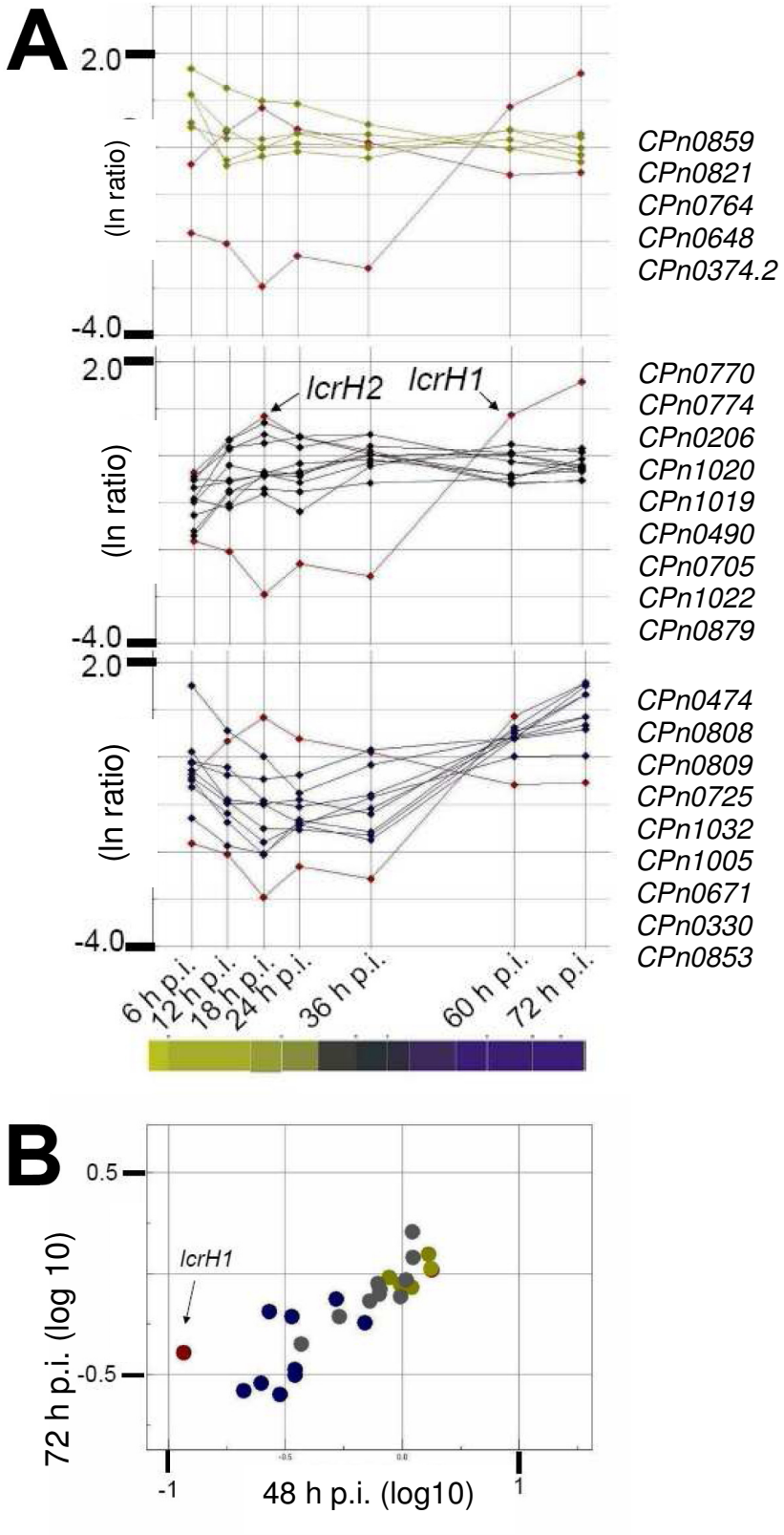


Figure 20 - **Regulation of predicted putative TTSS genes** The expression profiles of 24 genes coding for predicted TTSS proteins (Subtil, A. *et al.*, 2005c) as well as the two TTSS chaperones *IcrH1* and *IcrH2* (in red) are displayed. (A) According to the expression profiles in the acute developmental cycle the genes can be grouped into early, mid and tardy genes. Mid genes showed a correlated expression profile to *IcrH2*, whereas tardy genes showed a correlated expression profile to *IcrH1*. This indicates time dependent functions throughout the cycle. Data is represented as ln(ratio) (y-axis) and time points (x-axis). (B) Compare plot showing the expression of these 24 genes coding for the predicted TTSS proteins in the iron depletion-mediated persistence. The x-axis represents 48 h p.i. and the y-axis 72 h p.i. of the persistent infection. Mainly tardy genes are down-regulated. Data is displayed as log10. Gene identification numbers, gene definitions and gene names can be consulted at http://www.stdgen.lanl.gov.

3.2.4.3 Transport and energy metabolism

Chlamydiae are not able to synthesize purines, pyrimidines and several amino acids (Stephens, R. S. *et al.*, 1998c) and therefore rely on the import of a variety of host cell intermediates. Consistent with this a considerable number of genes encoding different transport proteins have been identified in the chlamydial genome, including ABC transporters. Figure 18 depict the expression profiles of predicted transporter homologous (http://www.genome.ad.jp/kegg) and their regulation during acute infection. The expression of genes encoding the transmembrane and ATP-binding proteins peaked early during the cycle of development, whereas genes for substrate binding proteins have different profiles. For instance, *artJ*, coding for an L-arginine-binding protein, is an 'Early' class gene whereas *fliY*, coding for a predicted glutamine-binding protein, is a 'Late' class gene.

It has been discussed that Chlamydiae are unable to alter the expression of metabolic genes in response to environmental changes (Iliffe-Lee, E. R. *et al.*, 2000;Nicholson, T. L. *et al.*, 2004). However, the data showed that metabolic pathways are differentially regulated throughout the infection. Early in the cycle (6 h p.i.) fructose-bisphosphate aldolase (*Cpn0281*) and *pgk* (*Cpn0679*), both involved in glycolysis, were expressed at high levels (10-fold for *Cpn0281* and 4-fold for *pgk*). Moreover, *tpiS* (*Cpn1063*), resulting in glyceronephosphate as an intermediary product, showed up-regulated expression. Glycerol-3-phosphatase acyltransferase (*Cpn0958*), *plsC* (*Cpn0569*) and *pssA* (*Cpn0983*), involved in the synthesis of phosphatidylglycerophosphate (PGP) exhibited the same early expression pattern. Another branch of this pathway, *pgsA1* (*Cpn0615*) and *psdD* (*Cpn0839*), leading to the synthesis of phosphatidylethanolamine (PE) also showed an early expression pattern. Both PGP and PE are essential membrane components (Suzuki, M. *et al.*, 2002). The genes *sdhB*, coding for the iron-sulfur subunit of succinate dehydrogenase, and *sdhC*, coding for apocytochrome b558, were both 4-fold up-regulated at early time points. Moreover *atpE* was 4-fold up-regulated at 6 h p.i. followed by 2-fold up-regulation of *atpA*, *atpB*, *atpE* and *atpI* at 12 h p.i. This reflects the pathogen's need for energy immediately after infection with glycolysis and oxidative phosphorylation being the main energy source. At subsequent stages of the cycle, changes in the transcriptome signature reflect different energy requirements: *gapA* (*Cpn0624*), *pgmA* (*Cpn0863*) and *eno* (*CPn0800*) are genes of clusters 8 and 9, leading to the synthesis of phosphoenolpyruvate that is fed into the citrate (TCA = tricarbone) cycle. Malate dehydrogenase (*Cpn01028*), *fumC* (*Cpn1013*), *sucC* (*Cpn0973*), *sucB1* (*Cpn0377*) and *lpdA* (*Cpn0833*) are also genes of the 'Late' cluster class. Chlamydiae are thought to depend on exogenous acquisition of 2-oxoglutarate which is fed into the chlamydial TCA cycle via OmpB (Kubo 2001). It is likely that these reverse

reactions support glycogenesis and the pentose phosphate pathway. The observation that genes involved in the pentose phosphate pathway, e.g. *zwf* (*Cpn0238* - cluster 5), *rpe* (*Cpn0185* - cluster 7) and *tal* (*Cpn0083* – cluster 8), are expressed in the 'Mid' to 'Late' class supports this hypothesis. The glucose-6-phosphate isomerase *pgi* (*Cpn1025*), a dimeric enzyme catalyzing the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate, is a tardy gene. In most organisms it is involved in glycolysis and in some bacteria it comprises a gateway for fructose into the Entner-Doudoroff pathway.

The expression patterns of these pathways showed that throughout the developmental cycle the requirement for energy changes. Whereas at early time points glycolysis and ATP production are the focal elements, later the acquisition of host intermediates seems to gain increasing importance for the maintenance of the developmental cycle.

3.2.4.4 Transcriptional and translational regulation

The translational machinery is mostly conserved among the different chlamydial species, and besides ribosomes, mRNA and tRNA, it requires aminoacids (AA), aminoacyl-tRNA and translation factors. Genes involved in tRNA amino-acylation include *metG*, *gltX*, *asps* expressed early, and *pheT*, expressed late, whereas translational initiation factors are mid genes with a maximum of expression between 6 h and 24 h post infection. Since the translational machinery is activated right after infection, this shows that the translational control is scattered throughout the developmental cycle.

Transcription is controlled at several key points: the α (*rpoA*) and β (*rpoB*) subunits of the RNA polymerase (Kalman, S. *et al.*, 1999c;Stephens, R. S. *et al.*, 1998b), as well as the major sigma factor rpoD/σ^{66}/σ^{B} and the alternative factors rpsD/σ^{28}/σ^{F} and rpoN/σ^{54}/σ^{N}, are responsible for the regulation of mid and late genes (Studholme, D. J. *et al.*, 2000;Mathews, S. A. *et al.*, 1999c;Douglas, A. L. *et al.*, 2000d). The major sigma factor was constantly expressed but decreased 1.8-fold at 72 h p.i.

The alternative sigma factor *rpoN* showed a constant expression. The activity of RpoN has been shown to be controlled by the two component system AtoC / AtoS in *E. coli* (Lioliou, E. E. *et al.*, 2004a). The homologous genes in Chlamydiae are named *ctcB* / *ctcC* (*Cpn0586* / *Cpn0548*). It has been discussed that CtcC has the potential to enhance transcription from sigma-54 specific promoters after being phosphorylated by CtcB (Koo, I. C. *et al.*, 2003), a sensor histidine kinase located in the plasma membrane in *E. coli* but lacking transmembrane domains in *Cpn* (Reitzer, L. *et al.*, 2001). The observation that *ctcB* and *ctcC* are ‘Late’ class genes (cluster 12) suggests that, upon appropriate stimulation,

enhanced transcription from sigma-54 specific promoters might be dependent on this two component system.

The expression of the second alternative sigma factor *rpsD* increased more than two-fold at the end of the cycle, suggesting a role for transcription of late and tardy genes. The genome also encodes *rsbW*, *rsbV1*, *rsbV2*, *rsbU* and *CPn0792*, all predicted regulators of RpsD activity (Douglas, A. L. *et al.*, 2000c). The latter two represent PP2C-like phosphatases with membrane spanning regions positioned to sense signals in the host cell environment. RsbW is an anti-sigma factor inactivating σ^B in *B. subtilis*. This interaction is controlled by the anti-σ factor RsbV and indirectly by its antagonist RsbU. One important stress signal of RsbU-RsbV-RsbW regulation is a decrease in the ATP level in the host (Hughes, K. T. *et al.*, 1998). The regulators *rsbW*, *rsBV2* and *rsbU* are mid to late genes, whereas *rsbV1* and *Cpn0792* was expressed constantly throughout the developmental cycle. This suggests different roles of RsBV1/RsBV2 and RsbU/*CPn0792* in regulating RsbW (van Schaik, W. *et al.*, 2005). However, a recent publication did show that RsbW alone is not sufficient to regulate RpsD activity *in vitro* (Hua, L. *et al.*, 2006).

3.2.5 Discussion

To survive within eukaryotic cells Chlamydiae must confront defense mechanisms and acquire metabolites necessary for bacterial growth. Two premises make the transcriptome data very attractive for tracking metabolic pathways and genes leading connected with morphological changes throughout the developmental cycle. First, intracellular pathogens, face a high evolutionary pressure, and therefore posses compacted genomes with protein synthesis immediately coupled to transcriptional events (Gowrishankar, J. *et al.*, 2004). Second, throughout the chlamydial developmental cycle each and every gene must be regulated at a certain point, as the EB have no active transcription. This allows concluding which processes play a central role at which stage of the developmental cycle. Therefore, microarray analysis of *Cpn* was performed to identify transcriptional changes at various stages of the developmental cycle. This led to the definition of 12 clusters and 4 main transcriptional stages termed as 'Early', 'Mid', 'Late' and 'Tardy' cluster classes. While existing literature mainly uses the onset of transcription for gene classification (Belland, R. J. *et al.*, 2003h;Nicholson, T. L. *et al.*, 2003b), this study used the gene expression profiles to characterize the *Cpn* transcriptome throughout the developmental cycle. Using this strategy enabled the description of time specific functions of genes. As an example this is the case for *incC,* coding for an inclusion membrane protein which has been grouped to be an early gene for *Ctr* (Nicholson, T. L. *et al.*, 2003a) because of its expression onset. In contrast in this study it was grouped among the 'Mid' class clusters because *incC* showed an expression peak at 18 h p.i. This classification of *incC* as a mid cycle gene corresponds much better to the morphological changes occurring throughout the developmental cycle of *Cpn*, as the growth of the inclusion size has been described to take place at mid cycle (Hammerschlag, M. R., 2002d). Additionally, genes coding for the transcriptional and translational machinery were connected to 'Mid' clusters showing defined peaks between 12 h p.i. and 24 h p.i., marking the time point of increased gene transcription and binary fission. Interestingly, the comparison to an EB proteome study revealed a significant correlation between 'Late' clusters and EB proteins. This is a noteworthy finding, as previously it was speculated that mostly genes showing the highest expression values at the end of the cycle would contribute to EB proteins. Therefore, the 'Tardy' gene cluster class was defined as distinct from the 'Late' class, based on its expression profile, the missing significant correlation to the EB protein content and the significant connection to EB mRNA. The *Cpn* transcriptome showed a remarkable correlation to the different stages of the acute infection. Gene expression therefore paves the way for different requirements of the pathogen during the developmental cycle. Genes were identified that play crucial roles as key factors giving insight into the energy metabolism, the biochemical composition and

the *Cpn* regulation of transcription during the acute infection. Proteins exposed on the surface are primary mediators in pathogen infection and transmission. The *Cpn* genome encodes a family of 21 polymorphic membrane proteins (pmp), with predicted outer membrane localization (Kalman, S. *et al.*, 1999b) which are discussed to be involved in adhesion, molecular transport and signaling (Henderson, I. R. *et al.*, 2001;Grimwood, J. *et al.*, 2001b;Wehrl, W. *et al.*, 2004b). Therefore it is of great interest, whether they are simultaneously expressed or how their expression is regulated during the *Cpn* developmental cycle. Genes coding for the family of polymorphic membrane proteins (pmps) showed time specific expression throughout the cycle. The finding that only four genes coding for pmps belong to early clusters, whereas 16 detected genes coding for pmps belong to late and tardy clusters suggest a different role in the membrane composition at different time points. As at later time points RB to EB re-differentiation occurs, this led to the speculation that the latter have a specific role in the re-differentiation process. Chlamydiae possess only *ftsY* and *ftsW*, but not *ftsZ* present in *E. coli* (Rothfield, L. *et al.*, 1999;Bramhill, D., 1997). Therefore, a role for peptidoglycans has been discussed not only in the transition from RB to EB prior to their release, but also in RB division (Chopra, I. *et al.*, 1998a). Of the peptidoglycan genes *murE* was grouped in the 'Early' cluster class, whereas *murA*, *murC*, *murD* and *murF* were grouped in the 'Late' cluster class. Genes involved in cell division, like *ftsY* and *ftsW* had highest expression mid cycle, and therefore belong to the 'Mid' cluster class. This indicates that an involvement of *murE* in binary fission might be possible, whereas the remainder of the peptidoglycan genes more likely play a role for RB to EB re-differentiation. Further, the expression patterns of *parB*, a gene of the 'Late' class, and *rodA*, which showed an expression with an early and a late peak, suggest a role in RB and in EB synthesis. Chlamydiae have long been expected to be energy parasites, unless truncated, energy and metabolic pathways have been detected (Stephens, R. S. *et al.*, 1998a;Kalman, S. *et al.*, 1999a). The expression patterns of metabolic pathways showed that throughout the developmental cycle the requirement for energy changes and genes belonging to the glycolysis (*dhn*, *pgk*) are up-regulated early, whereas genes belonging to the citrate cycle were up-regulated later in the developmental cycle. It has been discussed that Chlamydiae are able to import 2-oxoglutarate (Kubo, A. *et al.*, 2001a;Bavoil, P. M. *et al.*, 2000) and therefore are able to fuel the citrate cycle with host cell metabolites. Interestingly, also the pathway leading to the production of phosphatidylphosphoglycerate and phosphatidylethanolamine were up-regulated at early time points. These are essential membrane components and their early up-regulation therefore suggests that *Cpn* is able to synthesize them on their own. This indicates that at early time points glycolysis and ATP production are the focal elements, whereas later the acquisition of host intermediates is gaining increasing importance for the maintenance of

chlamydial growth. Secretion processes are important for the chlamydial survival in the host cell. *Cpn* possesses a Type Two (TIISS) and a Type Three Secretion System (TTSS) (Lugert, R. *et al.*, 2004) which allows them to target effector molecules into the host cell (Fields, K. A. *et al.*, 2005). Inhibition of apoptosis, alteration of tyrosine phosphorylation and degradation of transcription factors to suppress MHC class I and II expression have been already reported (Fan, T. *et al.*, 1998a;Heuer, D. *et al.*, 2003a;Zhong, G. *et al.*, 2001a;Fawaz, F. S. *et al.*, 1997a;Zhong, G. *et al.*, 1999;Zhong, G. *et al.*, 2000) to be connected with pathogen protein secretion. Since an intense crosstalk between the pathogen and the host is essential for these modulations, type III secreted proteins are likely to be involved in these processes. The data presented in this work showed that the TTSS system is transcribed late in the cycle, what is in consensus with the fact that EB possess a TTSS (Matsumoto, A., 1981). Therefore it has been discussed that the TTSS also plays a role in the infection process at the beginning of the cycle. Interestingly, the two chaperones *lcrH1* and *lcrH2*, necessary for the secretion process, showed a peak expression at different time points, therefore suggesting different roles at the beginning and the end of the cycle. This supports data from Slepenkin et. al. (Slepenkin, A. *et al.*, 2003a), who also addressed this question. The sec dependent TIISS is expressed in the middle of the cycle. Therefore different roles of the secretion systems throughout the cycle of development can be suggested.

Taken together this data provide an insight into the transcriptome of this intracellular pathogen *Cpn* leading to a deeper understanding of mechanisms involved in the progression of the developmental cycle. Beside identified pathways the *Cpn* genome contains a vast amount of hypothetical genes and time specific functions in the developmental cycle can easier be addressed taking the expression profiles into account.

3.3 Transcriptional changes in the iron depletion-mediated persistent infection

To analyze iron-starvation mediated persistence, a global transcriptome analysis of *Cpn* infections treated with deferoxamine (DAM) was performed. Iron-starved, persistent *Cpn* showed an altered morphological appearance (Figure 12), and it has been discussed that in the models of persistence Chlamydiae have a unique transcription profile with major functions being altered at a transcriptional level (Hogan, R. J. *et al.*, 2003b). Total RNA extracted from DAM-treated samples taken at 24 h, 48 h and 72 h p.i. was compared to corresponding time points of the acute infection. For iron repletion experiments, DAM-containing medium was replaced at 72 h p.i. with normal growth medium. Samples were taken 24 h and 48 h after the repletion time point and compared to 48 h p.i. of the acute infection). DAM-mediated persistence revealed an altered gene expression pattern as compared to normal development. Statistical analysis using SAM 2.0 identified 461 significantly regulated genes with 73 genes (FDR = 1.64%), 191 (FDR = 18.6%) and 311 (FDR = 1.33%) being significantly regulated at 24 h, 48 h and 72 h post infection (Figure 21 to Figure 24) (Supplementary-Table 3).

241 genes were differentially regulated more than 1.8-fold. Of these, 123 genes were down-regulated and 124 genes were up-regulated from 24 h p.i. to 72 h post infection. Clustering of significantly regulated genes using a self organizing map (SOM) displays two main clusters of regulation (Figure 25). At 24 h p.i. eight genes showed differential expression of which seven genes were down-regulated and only one gene was up-regulated. The down-regulated genes included *hctB*, *omcB* and *lcrH1*. At 48 h p.i. additionally 98 genes were up- and 82 down-regulated, whereas at 72 h p.i. additionally 25 genes were up- and 33 genes down-regulated.

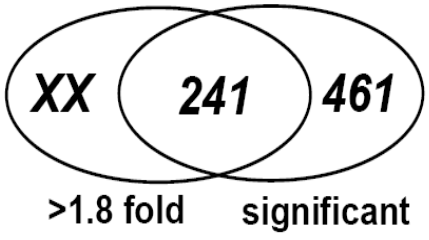


Figure 21 – **Total number of significant genes in the persistent infection.** 241 out of 461 significant calculated genes in the DAM induced persistent infection are regulated more than 1.8-fold.

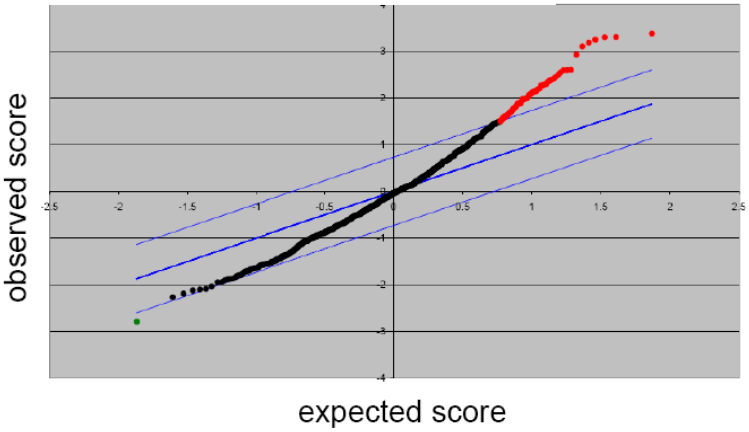


Figure 22 - **SAM analysis of the persistent infection at 24 h p.i.** Using Significant Analysis of Microarrays (SAM) 73 genes were calculated as significantly expressed at 24 h p.i. The False Discovery Rate (FDR) was 1.64%. The x-axis shows the expected score, the y-axis the observed score. Significant genes down-regulated are indicated in green, significant genes up-regulated are indicated in red.

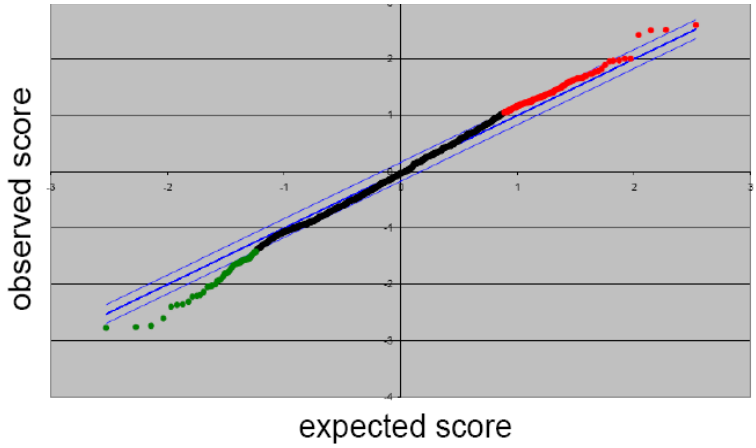


Figure 23 - **SAM analysis of the persistent infection at 48 h p.i.** Using Significant Analysis of Microarrays (SAM) 191 genes were calculated as significantly expressed at 48 h p.i. The False Discovery Rate (FDR) was 18.6%. The x-axis shows the expected score, the y-axis the observed score. Significant genes down-regulated are indicated in green, significant genes up-regulated are indicated in red.

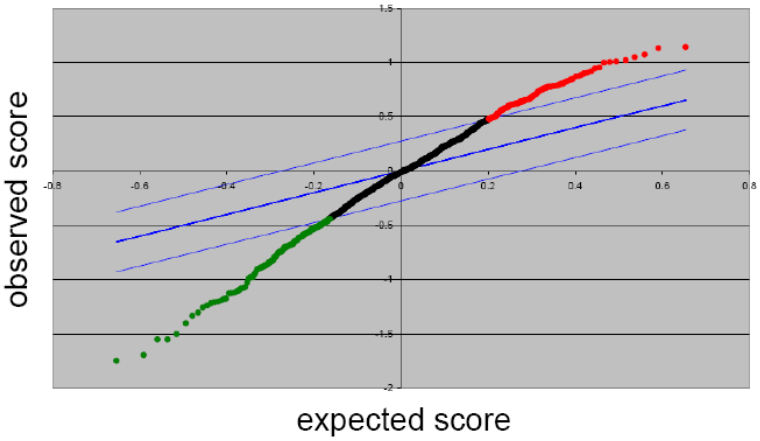


Figure 24 - **SAM analysis of the persistent infection at 72 h p.i.** Using Significant Analysis of Microarrays (SAM) 311 genes were calculated as significantly expressed at 72 h p.i. The False Discovery Rate (FDR) was 1.3%. The x-axis shows the expected score, the y-axis the observed score. Significant genes down-regulated are indicated in green, significant genes up-regulated are indicated in red.

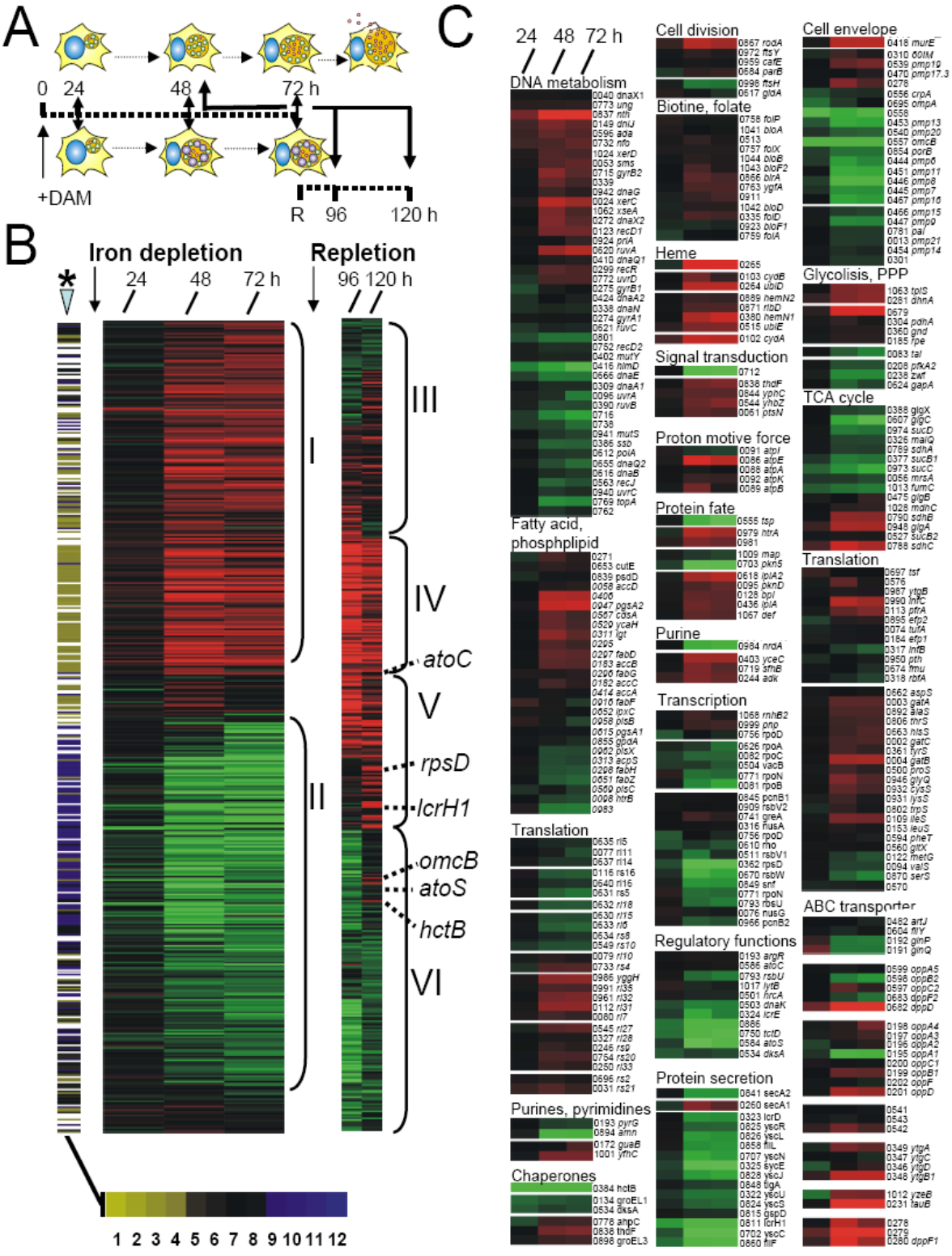


Figure 25 - **Differentially regulated genes during DAM-mediated iron persistent infection.** (A) Experimental design. Cells were infected with *C. pneumonaie* at time point 0, incubated with DAM at 2 h p.i. to obtain a persistent infection (+DAM) and lysed at 24, 48 and 72 h p.i. Iron-depleted cells were repleted at 72 h p.i. (R) and harvested at 96 h and 120 h p.i. Iron-depleted samples were compared with the according time points. Iron-repleted time points were compared with 48 h p.i. of the acute infection (indicated by arrows). (B) Clustered expression profiles of 461 significantly expressed genes calculated with SAM using the color scale according to Figure 17. Bars on the left indicate genes also significantly regulated during the acute infection with clusters marked (+) using the color bar below. (C) Functional grouping and detailed expression profiles of genes. Genes differentially expressed during persistent infection are displayed. For expanded annotated details see Supplementary Tables 2B,C,D and 3B. Gene identification numbers, gene definitions and gene names can be consulted at http://www.stdgen.lanl.gov.

3.3.1 Iron-mediated persistence is an arrest of transcription at mid stages of the acute infection

The nature of chlamydial persistence is still a matter of debate (Schachter, J. et al., 2002). To examine which gene clusters of the cycle are influenced most by iron depletion, the data from the developmental cycle and the persistence were compared. Out of 241 significantly and differentially regulated genes of the persistent infection 204 genes are present in the 754 significant regulated genes of the acute infection. Of these 204 genes 97 showed an explicit up-regulation and 104 genes an explicit down-regulation during persistence. The bar in Figure 17 displays the genes up- and down-regulated in persistence with green representing down- and red up-regulation. Of 97 genes significantly up-regulated during the persistent infection 54 were linked to cluster 2 ($n \geq 54$; $p < 0.01$) and 17 to cluster 3 ($n \geq 17$; $p < 0.01$) of the acute cycle. 104 genes were significantly down-regulated during the persistent stage with 24 linked to cluster 10 ($n \geq 24$; $p < 0.01$), 31 to cluster 11 ($n \geq 31$; $p < 0.01$) and 19 to cluster 12 ($n \geq 19$; $p < 0.01$) of the acute infection. Only cluster 8 was minor but not significantly affected, with down-regulation of 12 genes ($n \geq 12$; $p = 0.53$). No other clusters of the acute infection showed significant linkage to genes affected in persistence (Table 3 and Table 4). Genes up-regulated during the persistent infection were therefore associated to the cluster 2 and cluster 3, whereas down-regulated genes were associated to clusters 10, 11 and 12 (Figure 26). This suggests that persistence is not a completely new transcriptional profile but more likely an arrest at some point in the transcription of the cycle of development. Several genes differentially regulated in the persistence fit into acute mid cycle clusters. This included *rpoB*, the ß-subunit of the RNA polymerase, the *fmt* methionyl tRNA formyltransferase, and *tauB*, *nlpD*, *rl31*, *Cpn0798*, *secA1*, *ygfU*, *gatB*. However, the expression profiles resembled their profiles at 36 h p.i. of the acute cycle. This strengthened the notion that persistence is a transcriptional arrest in the acute mid cycle. It is still open whether the transcriptional profile seen during the DAM-mediated persistence results from iron depletion in the host cell, leading to a loss of signal for switching off early and switching on late and tardy genes, or from a reduced iron level inside the pathogen itself.

3.3.2 Predominant changes in the transcriptional pattern of the iron depletion-mediated persistent infection

Several genes coding for membrane-related proteins were differentially expressed in the persistent infection. Of the polymorphic membrane protein family only *pmp19* showed up-regulation in the persistent infection, whereas eleven genes of this family were down-regulated. Also *omcA* and *omcB*, described to be expressed late in the acute cycle, showed a down-regulated profile in the persistence. Of genes involved in peptidoglycan synthesis, *murB* was down-regulated, whereas *murE* showed up-regulation. Also *nlpD*, a cell wall-binding protein, and *CPn0278*, an outer membrane lipoprotein, showed up-regulation. The rod-shape determining protein *rodA* was 2-fold up-regulated. It was speculated that RodA is responsible for the abnormal chlamydial forms during the persistent infection (Henriques, A. O. et al., 1998). For other genes involved in cell division only *ftsH* showed a down-regulation, whereas the remainders were not altered. Further the secretion machineries were affected in the persistent infection. Genes responsible for the TIISS and TTSS were down-regulated, even 9-fold for the chaperone *lcrH1*. Of the putative 24 genes coding for TTSS proteins (Subtil, A. *et al.*, 2005a) only the group connected to tardy clusters was down-regulated in persistence (Figure 20). Only *secA*1, coding for a component of the TIISS, was up-regulated. The existence of two homologous genes coding for Sec proteins in the *Cpn* genome is unusual and only shared with a few other pathogenic bacteria (Braunstein, M. et al., 2001). It has been shown that SecA homologous can have different functions (Braunstein, M. et al., 2003) and might account for the investigated different expression profiles. 13 genes involved in energy metabolism were differentially expressed during persistence. The ATP synthase subunit *atpE* was increased up to 3-fold at 48 h post infection. Three genes involved in glycolysis (*tpiS*, *pkg* and *dhnA*), two genes of the oxidative phosphorylation (*sdhB*, *sdhC*) and one gene of starch and sucrose metabolism (*glgA)* were also up-regulated. In contrast, genes of the pentose phosphate pathway (zwf; tal), the TCA cycle (*sucC*) and two genes of starch and sucrose metabolism (*glgC*, *glgP*) were down-regulated.

Iron depletion-mediated changes in the transcription profile might have multiple reasons. DNA binding proteins including *himD*, a histone-like DNA binding stress response protein, and the histone-like protein *hctB* showed reduced transcription, whereas the expression of *hctA* was unchanged. Also the sigma (σ) factors and their regulatory networks showed an altered expression profile. The expression of *σ54* was not affected while *σ28* was 2-fold down-regulated in persistence. The activation of the two component

system *ctcB* / *ctcC* (*atoS* / *atoC*) is discussed to be necessary for transcriptional activation of σ54 promoters (Lioliou, E. E. *et al.*, 2005c) and the decreased expression of *ctcB* might result in a reduced activation of the protein CtcB. Of the genes coding for the two kinases RsbV2 and RsbV1, involved in RsbW regulation, the former showed an increased and the latter an unchanged expression profile. *CT539* and *rsbU* coding for PP2C-like kinase also involved in RsbW regulation, showed decreased expression.

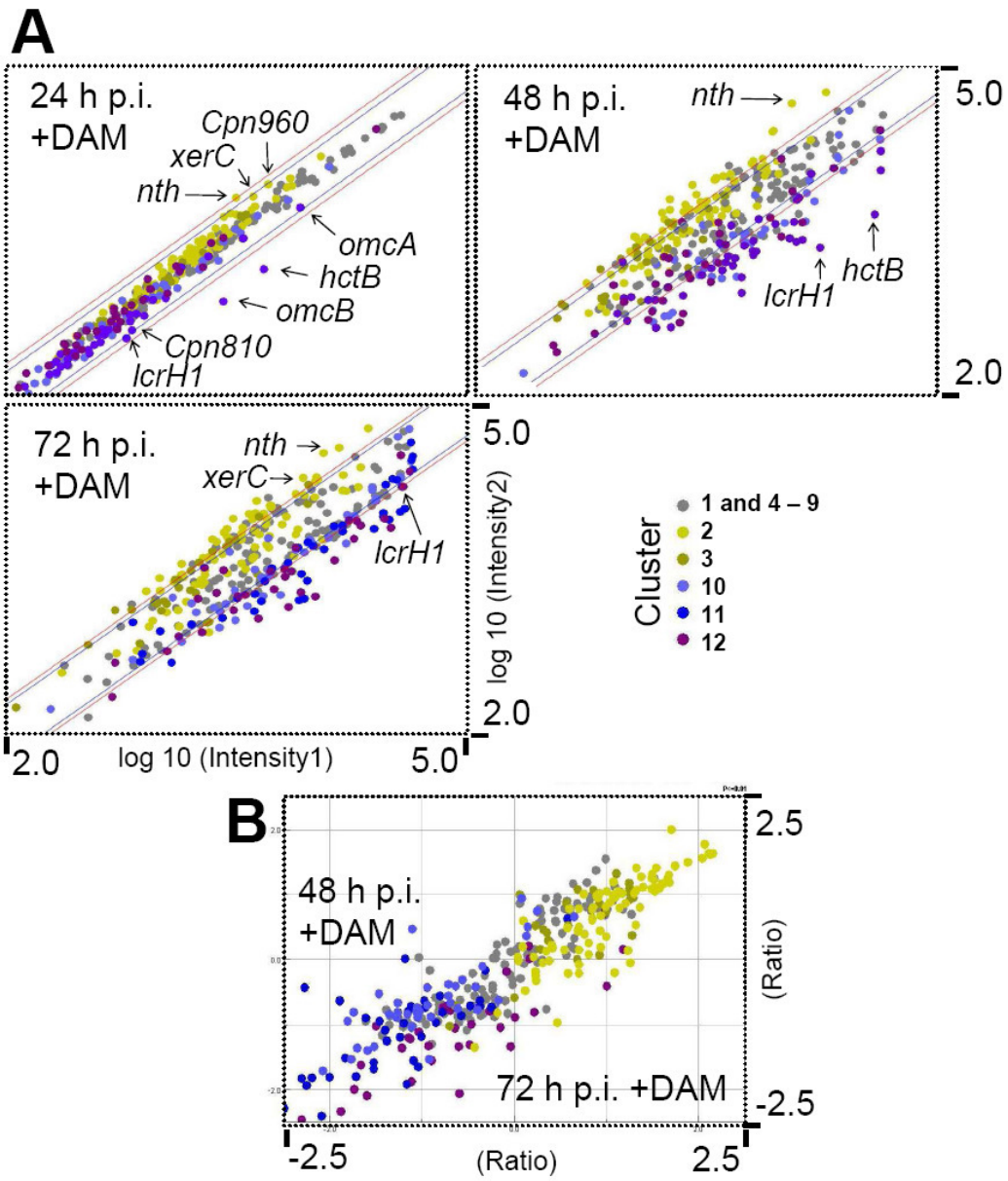


Figure 26 - **Intensity plot and compare plot of acute clusters significant down- or up-regulated in the persistent infection.** Only genes calculated as significantly expressed using SAM software in the persistent as well as in the acute infection are displayed. Genes linked to the acute clusters 2, 3, 10, 11 or 12 are highlighted. (A) Intensity plots for 24 h, 48 h, 72 h p.i. showing expression values of the persistent infection compared to the analogues time points of the acute infection. Data are displayed as log 10 values of the raw intensities. The blue and the red line indicate a 1.8 and a 2.0-fold cut off, respectively. (B) Compare plot of the ratio expression values of genes in the persistent infection at 48 h and 72 h p.i. Gene identification numbers, gene definitions and gene names can be consulted at http://www.stdgen.lanl.gov.

3.3.3 Transcriptome signatures of iron-repleted *Cpn*

In order to monitor gene expression changes during recovery of persistent bacteria the DAM-containing media was replaced with normal growth medium. Reactivation of *Cpn* correlates with an up-regulation of genes, including *rpsD*, *omcB*, *lcrH1*, *hctB* and *atoS*, belonging to the 'Late' gene cluster (group V, Figure 25). Some of the up-regulated genes from persistent *Cpn* were also up-regulated during iron repletion (group IV, Figure 25). However, the majority of genes that were up-regulated during persistence returned to expression levels similar to those in the late stages of the developmental cycle after iron repletion (group III, Figure 25). Group VI shows genes down-regulated in persistence and up-regulated again upon iron repletion. In summary, iron repletion led to a coordinated continuation of the developmental cycle.

Table 3 – **Comparison of significantly down-regulated genes in the persistent infection to the acute clusters.** The appearance of these genes according to the clusters of the acute infection was calculated using LACK software. The first column shows the cluster number of the acute infection, the second the size and the third the abundance of persistence induced downregulated genes for each cluster. The last column shows the calculated p-value for the abundance of persistence induced down-regulated genes. Down-regulated genes in the persistence were linked to cluster 10 to 12 of the acute infection.

Cluster #	Cluster Size	Hits	Complete Size	Full Dataset	p-Value
12	42	19	754	104	(p>=19)<0.01
11	48	31	754	104	(p>=31)<0.01
10	80	24	754	104	(p>=24)<0.01
9	50	0	754	104	(p>=0)=1.00
8	86	12	754	104	(p>=12)<0.53
7	34	3	754	104	(p>=3)=0.87
6	55	3	754	104	(p>=3)=0.98
5	65	6	754	104	(p>=6)=0.90
4	64	3	754	104	(p>=3)=0.99
3	53	1	754	104	(p>=1)=0.99
2	142	1	754	104	(p>=1)=0.99
1	34	1	754	104	(p>=1)=0.99

Table 4 - **Comparison of significantly up-regulated genes in the persistent infection to the acute clusters.** The appearance of these genes according to the clusters of the acute infection were calculated using SAM software. The first column shows the cluster number of the acute infection, the second the size and the third the abundance of persistence induced up-regulated genes for each cluster. The last column shows the calculated p-value for the abundance of persistence induced up-regulated genes. Up-regulated genes in the persistence were linked to cluster 2 and 3 of the acute infection.

Cluster #	Cluster Size	Hits	Complete Size	Full Dataset	p-Value
12	42	3	754	97	(p>=3)<0.92
11	48	0	754	97	(p>=0)=1.00
10	80	1	754	97	(p>=1)=0.99
9	50	7	754	97	(p>=7)=0.47
8	86	4	754	97	(p>=4)=0.99
7	34	1	754	97	(p>=1)=0.99
6	55	2	754	97	(p>=2)=0.99
5	65	3	754	97	(p>=3)=0.99
4	64	2	754	97	(p>=2)=0.99
3	53	17	754	97	(p>=17)=0.01
2	142	54	754	97	(p>=54)<0.01
1	34	2	754	97	(p>=2)=0.94

3.3.4 Discussion

It has been discussed that long-term or persistent chlamydial infections are implicated in chronic diseases (Worm, H. C. *et al.*, 2004). There is evidence that chlamydial species are capable of causing "enduring" infections in the absence of treatment (Moulder, J. W., 1971;Moulder, J. W. *et al.*, 1980). Several *in vitro* models are used to study persistent conditions, mainly IFNγ stimulation, treatment with antibiotics and iron depletion. Still there is not enough knowledge how Chlamydiae act in a persistent infection. Especially transcriptome changes are poorly described. The *C. trachomatis* transcriptome has been characterized for the acute infection and for the IFNγ persistence (Belland, R. J. *et al.*, 2003i;Belland, R. J. *et al.*, 2003a). However, for *Cpn* a whole transcriptome study for any of the persistence models has not been performed before. The transcriptome profile in the DAM mediated iron depletion persistence revealed an altered profile compared to the acute infection. 13 genes were identified as differentially regulated at 24 h p.i. and 241 genes identified differentially regulated at later time points (Figure 21). SAM-analysis revealed diverse False Discovery Rates (FDR) for these time points with 1.64%, 18.6% and 1.33% for 24 h, 48 h and 72 h p.i., respectively. Even though prominent genes are already influenced at 24 h p.i. the establishment of the persistent infection has not taken place yet. The histone-like protein *hctB* is 5-fold down-regulated at 24 h p.i. in the persistent infection. *HctB* transcription has been described to be sigma-*28* dependent (Yu, H. H. *et al.*, 2003b). The down-regulation of *omcA*, *omcB* and *lcrH1* and the up-regulation of *nth* at 24 h p.i. are also early indicators of the persistent infection. At 48 h p.i. the changed transcription profile indicated the establishment of the persistent infection compared to the acute infection. At 48 h p.i. the high false discovery rate (FDR) of 18.6% accounted for a high variance in the *Cpn* population. At 72 h p.i. the low FDR of 1.33% indicated a stable persistent population. The transcriptome of the iron depletion-mediated persistence was compared with the transcriptome profile of the developmental cycle. Genes up-regulated in the persistence were significantly linked to the genes of the acute clusters 2 and 3, whereas down-regulated genes in the persistence were significantly linked to genes of the acute clusters 10, 11 and 12. This indicated that the iron depletion-mediated persistence is not a new transcriptional profile. It is rather an arrest in gene expression at mid stages of the developmental cycle and it can be suggested that persistence establishes as a result of an arrest in the acute ‘transcriptome clockwork’. A possible explanation for this transcriptome arrest might be that iron starvation leads to missing signals for chlamydial transcriptome progression.

The down-regulation of *ctcB* being part of the two-component system *ctcB* / *ctcC* might be a key mediator in the establishment of persistence. It has been shown that this two-component system is involved in the transcriptional activation of sigma54 promoters (Hughes, K. T. *et al.*, 1998). The finding that clusters significantly contributing to the EB protein composition in the developmental cycle were not affected in persistence also is of importance (Cluster 8 ($n>=15$; $p=0.02$), cluster 9 ($n>=28$; $p<0.01$)*;* Table 2). Therefore it can be speculated that the absence of the RB to EB re-differentiation described for the iron depletion-mediated persistence (Al Younes, H. M. *et al.*, 2001a) is based only on a minority of genes connected to EB proteins. For the *Cpn* developmental cycle it was shown, that 16 genes coding for polymorphic membrane proteins (pmps) accumulate in the acute clusters 10 and 11. As genes of these clusters are significantly targeted in the iron depletion-mediated persistence, the down-regulation of pmps might have a key role for the development of persistence. Some studies already analyzed *C. pneumoniae* persistence by using qRT-PCR technology. Chlamydial signal transduction genes are differentially regulated in IFNγ induced persistent *Cpn* infections (Polkinghorne, A. *et al.*, 2005). In contrast to the IFNγ-model in the iron-depletion mediated persistence some genes revealed a different regulation. The gene *yphC*, linked to cell cycle control, was not differentially regulated, whereas *lytB* was down-regulated in DAM mediated persistence. The gene *htrA,* involved in bacterial stress response, was up-regulated in both the DAM mediated and the IFNγ stimulation persistence model. Also the question of differential expression of genes encoding membrane proteins has been addressed in a continuous infection model (Hogan, R. J. *et al.*, 2003a). This led to the identification of five up-regulated candidates (*CPn0483, nlpD, ompA, pmp1 and porB*). The DAM mediated persistence revealed only the up-regulation of *nlpD* coding for an *endopeptidase*, whereas the others did not show significant up-regulation. In contrast to a recent report for the IFNγ persistence (Byrne, G. I. *et al.*, 2001b), *ftsK* and *ftsW* were not altered in the iron depletion persistence model. Therefore, a part of the transcriptional changes observed seems to be specific for the iron depletion mediated persistence model and not be general for all persistence models. The alternative sigma factors have key roles for the regulation of the chlamydial genome. It has been shown that sigma-54 is important for the expression of mid to late genes and sigma-28 for late to tardy genes (Mathews, S. A. *et al.*, 1999b;Yu, H. H. *et al.*, 2003a;Shen, L. *et al.*, 2004c;Mathews, S. A. *et al.*, 2000;Douglas, A. L. *et al.*, 2000b). The *Cpn* genes *ctcB* and *ctcC* share significant homology the *E. coli* transcriptional activators *atoS* and *atoC* of the two component regulatory system family. This two component system is important for the

transcriptional regulation of sigma-54 promoters. It consists of a "sensor" kinase and a response regulator which often also is a transcriptional factor. By sensing an appropriate signal, the sensor kinase autophosphorylates and phosphorylates the response regulator, which leads to its activation (Lioliou, E. E. *et al.*, 2004b;Lioliou, E. E. *et al.*, 2005b). In other eubacteria AtoS is a membrane bound receptor sensing the bacterial environment (Lioliou, E. E. *et al.*, 2005a), whereas in Chlamydiae the AtoS homologue CtcB is likely to be a cytoplasmic protein without transmembrane domains. The data in iron depletion-mediated persistence showed that *ctcC* (*atoC*) is not changed during the persistent infection, whereas *ctcB* (*atoS)* is down-regulated 2-fold in the persistent infection. It can therefore be concluded that this two-component system is involved in the establishment of persistence. Also sigma-28, discussed to be important for the regulation of late genes (Shen, L. *et al.*, 2004b;Yu, H. H. *et al.*, 2003c) is 2-fold down-regulated in the persistent infection. A possible mechanism for the down-regulation of sigma-28 in persistent infections is discussed below.

Taken together, the data showed a remarkable connection between the acute and iron mediated persistent infection and provide insights into the mechanisms for the establishment of the iron depletion-mediated persistent infection.

3.4 Establishment of a housekeeping index for qRT-PCR normalization

Normalization of microarrays and qRT-PCR is based on different premises. While transcriptome data measure the expression of a huge amount of genes simultaneously, qRT-PCR only measures a single gene. Therefore normalization against an internal standard is required for qRT-PCR and usually ribosomal RNA (rRNA); total RNA or housekeeping genes that exhibit minimal variation of expression within the experimental setting are used. The purpose of these references is to remove or reduce differences due to sampling, i.e. differences in RNA quantity and quality. Most studies make use of control genes without validation of the supposed expression. Housekeeping gene expression, as well as rRNA molecules or total RNA, although often constant in a given cell type or experimental condition, still may show significant variation (Thellin, O. *et al.*, 1999;Solanas, M. *et al.*, 2001;Johnson, M. L. *et al.*, 1995;Warner, J. R., 1999;Warrington, J. A. *et al.*, 2000;Ryo, A. *et al.*, 2000;Bustin, S. A., 2000). The ideal endogenous reference should be at a constant level among the experimental set-up, e.g. the different tissues of an organism or all stages of a time course and should also be unaffected by the experimental treatment (Bustin, S. A. *et al.*, 2004). An increasing number of publications are discussing the selection of reference genes in qRT-PCR analyses (Radonic, A. *et al.*, 2004;Dheda, K. *et al.*, 2004). Therefore prior to qRT-PCR gene expression analysis the most stable housekeeping genes for a time course and special condition should be assigned using the e.g. geNORM software.

3.4.1 *ABCT*, *L29* and *tufA* are suitable housekeeping genes for *C. pneumoniae*

As no published data are available so far if 16S rRNA is a suitable housekeeping gene for qRT-PCR analysis of *Cpn* gene expression, its variation throughout the measured time points was compared with other housekeeping candidates. Ten genes were chosen from the 100 least regulated genes based on the microarray results of the acute and persistent infection. Particular emphasis was placed on genes that belong to different functional classes, reducing the risk that genes might be co-regulated. A gene stability measurement was carried out relying on the principle that the expression ratio of two ideal internal control genes is identical in all samples,

regardless of the experimental condition or cell type (Vandesompele, J. *et al.*, 2002). Real time PCR experiments for the acute infection and for the DAM mediated persistence were performed, and a ranking of these control genes was made according to their expression stability using the geNorm software (http://medgen.ugent.be/~jvdesomp/genorm) (Table 5). The M-value is the gene expression stability parameter as calculated by geNorm. The lower the M-value, the more stably expressed is the reference gene. For normalization of chlamydial gene expression, the 16S rRNA has been used in a variety of studies (Belland, R. J. *et al.*, 2003j), but was not identified amongst the best three candidates here. Subsequently, a house keeping index (HKI) was built with the geometric mean of the three most stable housekeeping candidates *ABCT*, *L29* and *tufA*. Additionally, *16S* rRNA was also used for normalization. So far, this approach has been applied for the first time for an intracellular bacterium.

In accordance to the microarray experiments, for the acute infection the 48h p.i. time point has been used and for the persistent infection the according time points have been used as reference. To verify the microarray results, qRT-PCR experiments were performed for randomly selected genes and genes of interest (GOI) from different regulated clusters. For calculation, the efficiency corrected ΔΔCT model (Pfaffl, M. W., 2001b) was used. Experiments were done at least in duplicate measurements using at least two biological replicates. A comparison of microarray and qRT-PCR results including 16S rRNA and HKI is presented in Figure 27 and Figure 28.

3.4.2 Discussion

The 16S rRNA showed a higher standard deviation and higher scattering compared to HKI especially at early and late time points possibly due to metabolic inactive EB also carrying 16S rRNA. Nevertheless, similar values were identified in relative gene expression independent of the applied method.

However, as expected, qRT-PCR generally produces higher values compared to microarray results. In the experiments performed in this work, the 16S rRNA showed a higher standard deviation and higher scattering compared to HKI. In this study similar values in relative gene expression independent of the applied method were identified.

Table 5 - **Determination and stability of housekeeping gene candidates together with chosen control genes.** The M-value has been calculated with GeNORM for every gene using the acute and persistent time points. The housekeeping candidates are ranked according to the calculated M-value, with higher M-values being less stable housekeeping genes. Gene identification numbers, gene definitions and gene names can be consulted at http://www.stdgen.lanl.gov.

Rank	Gene name	M-value
	lcrH1	1.76
	yscC	1.88
	sctS	0.92
	sycE	1.94
	sctN	1.6
#1	*pmp18*	1.53
#2	*groEL*	1.45
#3	*16S*	1.19
#4	*minD*	1.11
#5	*murB*	1.04
#6	*cdp*	0.86
#7	*rs2*	0.78
#8	*tufA*	0.71
#9	*L29*	0.59
#10	*ABCT*	0.59

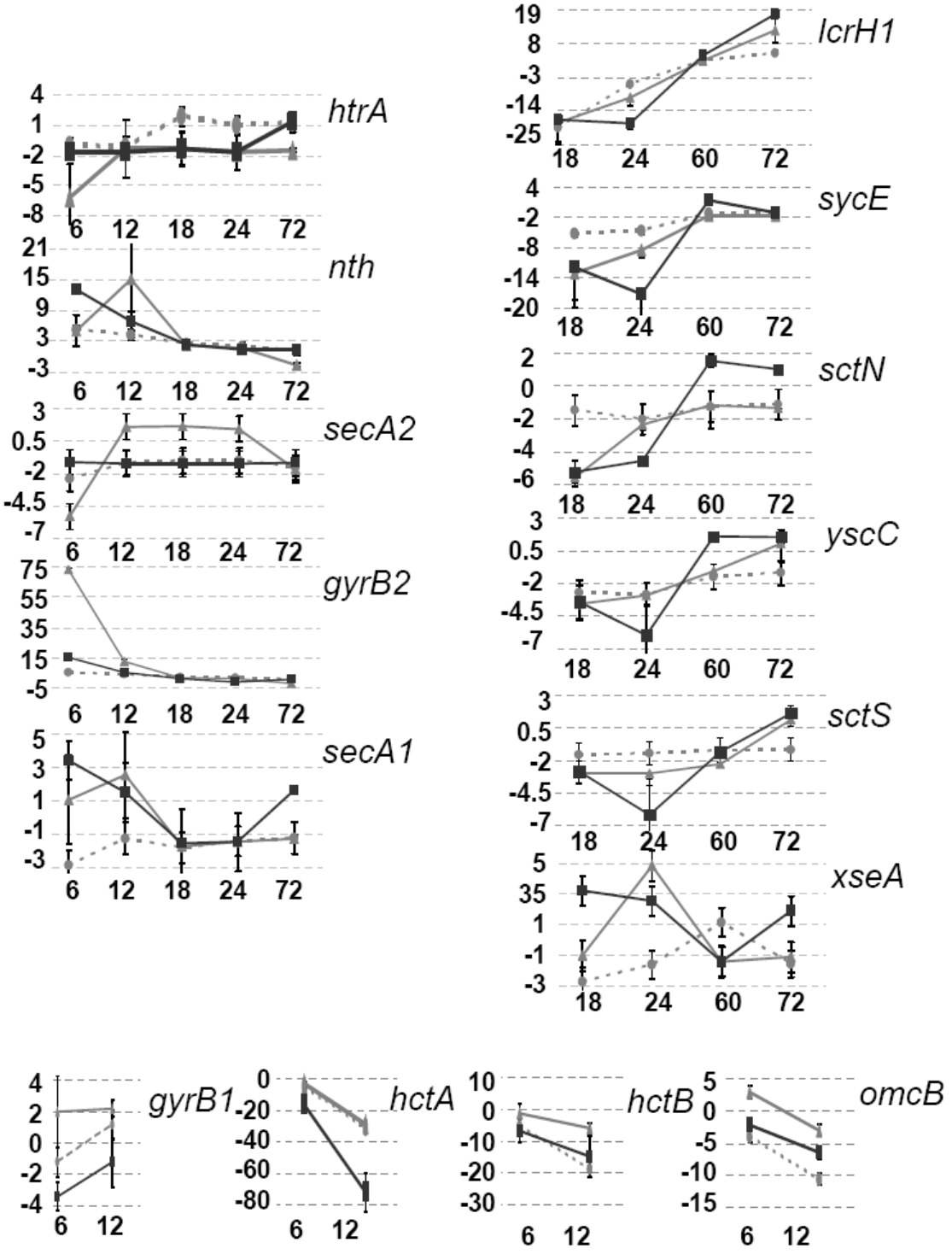


Figure 27 - **qRT-PCR verification of microarray data for differentially regulated genes of the acute infection.** Genes were picked randomly from different clusters. Normalization was done using HKI (black squares and solid black lines) and 16S rRNA (grey triangles and solid grey lines). Microarray expression data are depicted in solid grey dots and grey dashed lines. Error bars show standard deviation. The x-axis shows the time point post infection (in hours), whereas the y-axis shows the gene expression (in fold change). Gene identification numbers, gene definitions and gene names can be consulted at http://www.stdgen.lanl.gov.

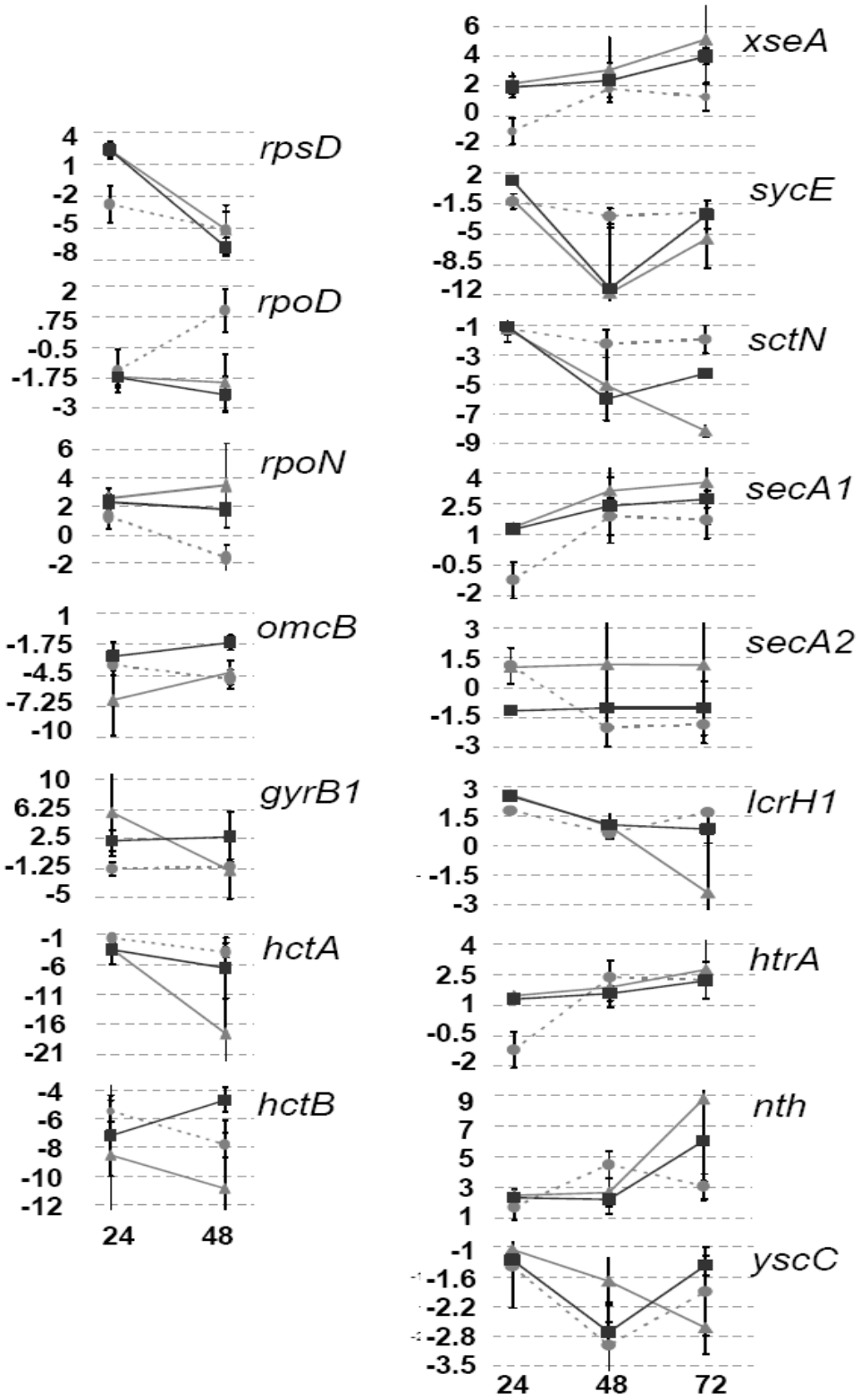


Figure 28 - **qRT-PCR verification of microarray data of differentially regulated genes of the persistent infection.** Genes were picked randomly from different clusters. Normalization was done using HKI (black squares and solid black lines) and 16S rRNA (grey triangles and solid grey lines). Microarray expression data are depicted in solid grey dots and grey dashed lines. Error bars show standard deviation. The x-axis shows the time point post infection (in hours), whereas the y-axis shows the gene expression (in fold change). Gene identification numbers, gene definitions and gene names can be consulted at http://www.stdgen.lanl.gov.

3.5 Gene orientation and gene expression

Genes can be oriented either tandemly (>> or <<), divergently (<>) or convergently (><) on a genome (Figure 29). It has been reported, that gene orientation can have an impact on the regulation of genes (Chiaromonte, F. *et al.*, 2003a). Intracellular bacteria face an enormous evolutionary pressure (Sakharkar, K. R. *et al.*, 2004b) to minimize genome length and intergenic regions (IGR). Therefore, it can be assumed that the gene orientation has an even more crucial influence on the transcription level compared to organisms with larger IGR.

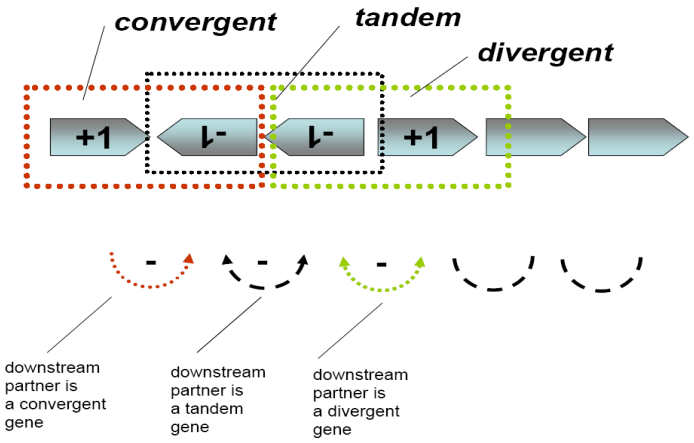


Figure 29 – **Tandemly, convergent and divergent oriented genes.** Genes can be oriented either tandemly (black square), divergent (green square) or convergent (red square) to their downstream neighbors.

3.5.1 Convergently and divergently oriented genes have an antagonistic expression profile at early stages of the acute infection

The genome of *Cpn* strain *CWL029* was screened for gene orientation and resulted in 754 tandemly, 149 divergently and 148 convergently oriented gene pairs. Out of these, 148 for the tandemly, 5 for the divergently and 44 for the convergently orientated genes even shared sequence information with their neighbour (Table 6).

Table 6 – **Amount of tandemly, divergently and convergently oriented gene pairs in the *Cpn CWL029* genome** (according to accessible ORFs at NCBI).

	tandemly oriented gene pairs (>> or <<)	divergently oriented gene pairs (<>)	convergently oriented gene pairs (><)
total number in *Cpn* genome	754	149	148
overlapping	148	5	44

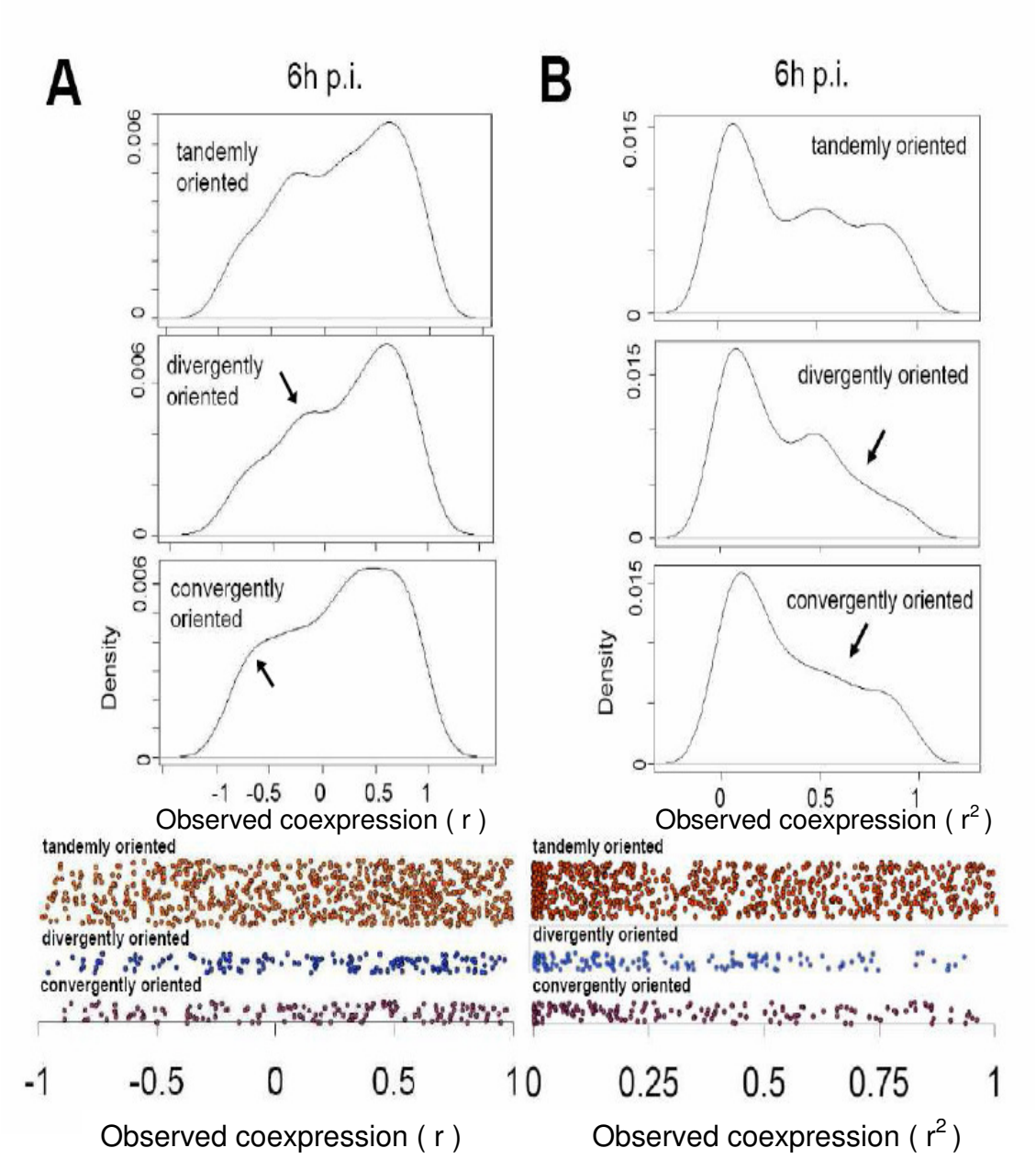


Figure 30 - **Correlation of gene expression of tandemly, divergently and convergently transcribed *Cpn* gene-pairs at 6 h p.i.** The distribution of the (A) Pearson correlation coefficient (r) and the (B) stability index (r^2) (square value of the Pearson correlation coefficient) is shown for 749 tandemly, 149 divergently and 147 convergently transcribed neighboring gene pairs. A low Pearson correlation coefficient (→ -1) counts for antagonistic coexpression, whereas a high Pearson correlation coefficient (→ 1) counts for correlated coexpression. A low stability index (→ 0) counts for no coexpression, whereas a high stability index (→ 1) counts for a correlated expression. The dot clouds display the correlation for individual gene pairs for which expression data was available. Dots were randomly positioned in y-axis direction to reduce the overlap. Above, density curves estimated from the dot clouds using Gaussian kernel density estimation are shown.

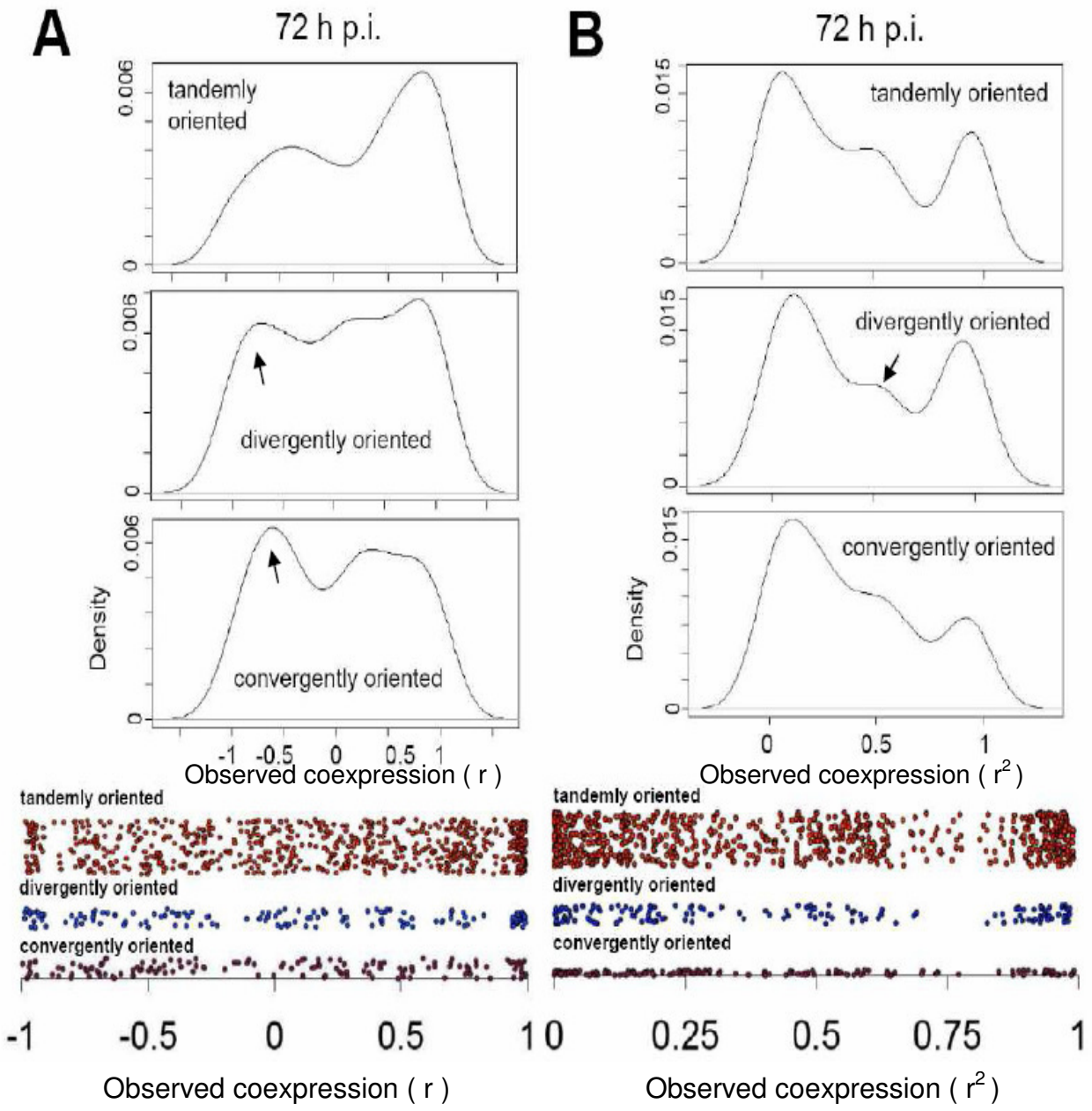


Figure 31 - **Correlation of gene expression of tandemly, divergently and convergently transcribed *Cpn* gene-pairs at 72 h p.i.** The distribution of the (A) Pearson correlation coefficient (r) and the (B) stability index (r^2) (square value of the Pearson correlation coefficient) is shown for 749 tandemly, 149 divergently and 147 convergently transcribed neighboring gene-pairs. A low Pearson correlation coefficient (→ -1) counts for antagonistic coexpression, whereas a high Pearson correlation coefficient (→ 1) counts for correlated coexpression. A low stability index (→ 0) counts for no coexpression, whereas a high stability index (→ 1) counts for a correlated expression. The dots clouds display the correlation for individual gene-pairs for which expression data was available. Dots were randomly positioned in y-axis direction to reduce the overlap. Above, density curves estimated from the dot clouds using Gaussian kernel density estimation are shown.

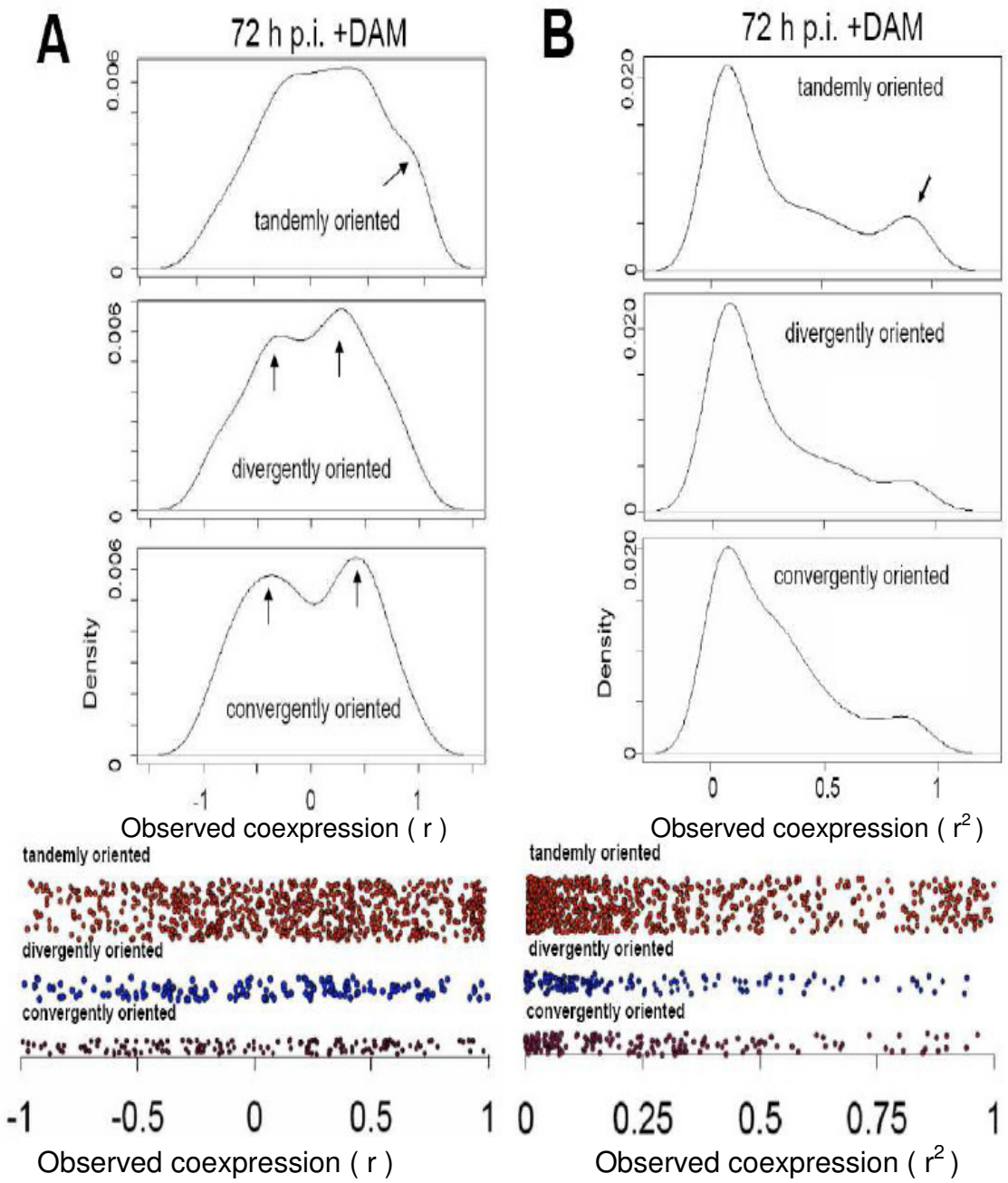


Figure 32 - **Correlation of gene expression of tandemly, divergently and convergently transcribed *Cpn* gene-pairs at 72 h p.i. in the deferoxamine induced persistent infection**. The distribution of the (A) Pearson correlation coefficient (r) and the (B) stability index (r^2) (square value of the Pearson correlation coefficient) is shown for 749 tandemly, 149 divergently and 147 convergently transcribed neighboring gene-pairs. A low Pearson correlation coefficient (→ -1) counts for antagonistic coexpression, whereas a high Pearson correlation coefficient (→ 1) counts for correlated coexpression. A low stability index (→ 0) counts for no coexpression, whereas a high stability index (→ 1) counts for a correlated expression. The dot clouds display the correlation for individual gene pairs for which expression data was available. Dots were randomly positioned in y-axis direction to reduce the overlap. Above, density curves estimated from the dot clouds using Gaussian kernel density estimation are shown.

The Pearson correlation coefficient (r) and the stability index (r^2) were calculated for gene pairs at 6 h p.i., 72 h p.i. of the acute infection and at 72 h p.i. of the persistent infection. A low Pearson correlation coefficient ($r \rightarrow -1$) counts for antagonistic coexpression, whereas a Person coefficient of $r = 0$ indicates no coexpression and a high Pearson correlation coefficient ($r \rightarrow 1$) correlated coexpression. Furthermore, a low stability index ($r^2 \rightarrow 0$) indicates no coexpression, whereas a high stability index ($r^2 \rightarrow 1$) counts for a correlated or anticorrelated coexpression. The calculation included 754 tandemly, 148 divergently and 147 convergently transcribed gene pairs for which expression data were available. Dot clouds were plotted for tandemly, divergently and convergently arranged gene pairs and the density curves estimated from the dot clouds using Gaussian density estimation.

At 6 h p.i. the stability index density curve (Figure 30) for tandemly oriented gene pairs showed three main groups. For the first no coexpression was observed ($r^2 \rightarrow 0$), while the latter two revealed higher coexpression ($r^2 \rightarrow 0.5$ and $r^2 \rightarrow 1$) with the Pearson correlation coefficient (Figure 30) showing mainly correlated expression ($r \rightarrow 1$). For divergently oriented gene pairs less coexpression ($r^2 \rightarrow 1$) was observed and consequently only two groups were present ($r^2 \rightarrow 0$ and $r^2 \rightarrow 0.5$) in the population. In contrast, for convergently oriented gene pairs a group with anticorrelated expression ($r \rightarrow -1$) was observed. At 6 h p.i. only slight differences were observed for tandemly, divergently and convergently oriented gene pairs, however at 72 h p.i. the picture changes (Figure 31). For all three populations three groups were present ($r^2 \rightarrow 0$; $r^2 \rightarrow 0.5$; $r^2 \rightarrow 1$) ranging from no coexpression to high coexpression, while among the convergently and divergently oriented gene pairs the amount of antagonistic expression ($r \rightarrow -1$) was much higher compared to tandemly oriented gene pairs. At 72 h p.i. in the persistent infection almost all genes showed no correlated expression ($r^2 \rightarrow 0$), while for the expression of convergently and divergently oriented genes two main groups, one with a correlated expression ($r \rightarrow -0.5$) and the other with anticorrelated expression ($r \rightarrow 0.5$) was observed (Figure 32).

3.5.2 Genes predicted for the *C. pneumoniae* strains *AR39* and *J138* also showed expression for the strain *CWL029*

Genome sequencing has led to an enormous amount of data for a variety of organisms. Still the prediction of genes stays difficult for a majority of genes. Predicted open reading frames (ORF) may turn out not to be correct, whereas also putative non coding regions include genes that might have been missed. Therefore, computational ORF predictions still need verification with experimental data to prove these predictions and microarray experiments offer an ideal method to verify and improve gene prediction methods. Genes for the *Cpn* strain *CWL029* have been forecasted by Stanford and UC Berkeley (http://www.stdgen.lanl.gov/stdgen/bacteria/cpneu/index.html). However, for the strains *AR39* or *J138* different algorithms have been used revealing in a slightly different set of predicted ORFs. Interestingly, these microarray studies done with the strain *CWL029* showed also signals for genes thought to be specific for *AR39* and *J138.* A genome blast performed with these genes in the *CWL029* genome detected these genes mostly in non-coding regions or overlapping with other genes (Figure 33 and Figure 34).

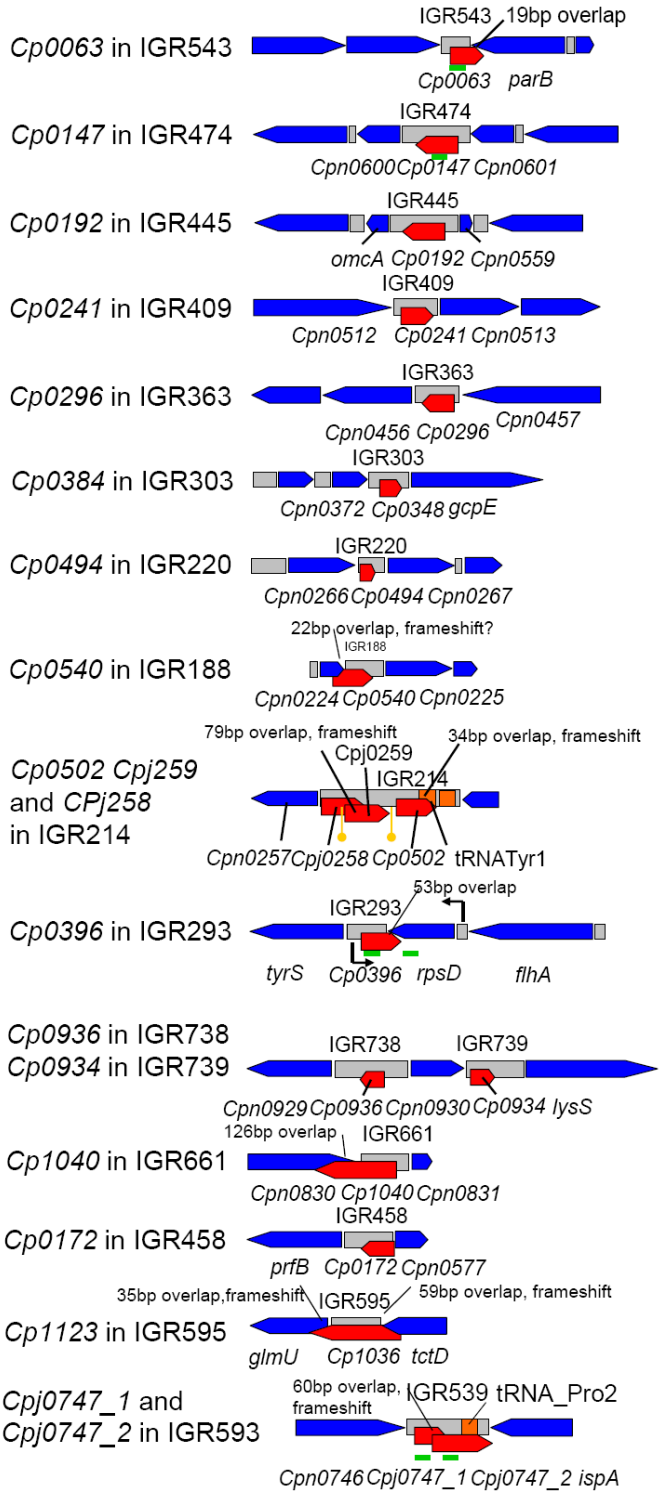


Figure 33 - **Schematic overview showing novel genes for *Cpn* strain *CWL029* (Part I)**. Pedicted genes for strain *CWL029* are displayed in blue, with intergenic regions in grey. Red arrows indicate genes showing predicted for the *AR39* or *J138* genome but showing expression in the acute or persistent infection experiments performed with *CWL029*. To locate these genes a blast has been performed revealing these genes in the *CWL029* genome. Green bars indicate the position of the oligos used, whereas black arrows show positions of putative promoters. Gene identification numbers, gene definitions and gene names can be consulted at **http://www.stdgen.lanl.gov** and NCBI.

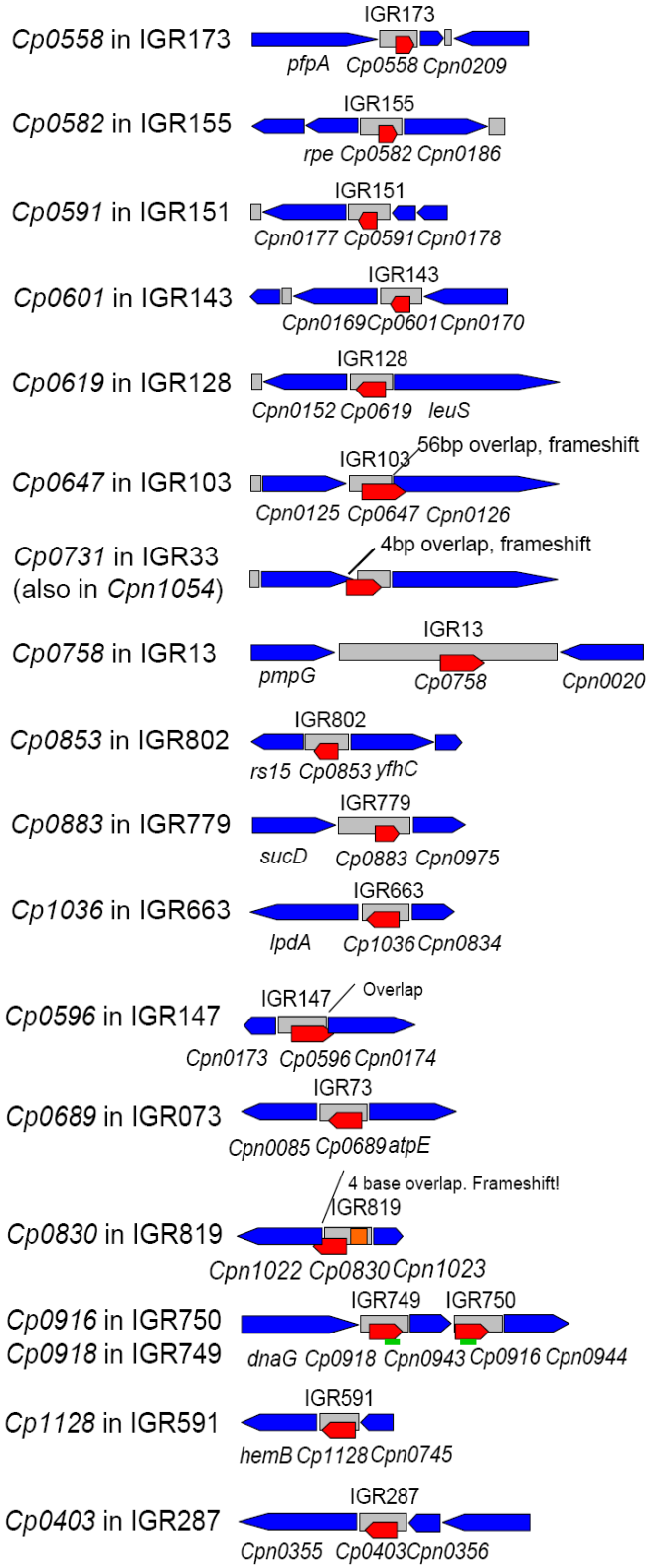


Figure 34 - **Schematic overview showing novel genes for *Cpn* strain *CWL029* (Part II)**. Pedicted genes for strain *CWL029* are displayed in blue, with intergenic regions in grey. Red arrows indicate genes showing predicted for the *AR39* or *J138* genome but showing expression in the acute or persistent infection experiments performed with *CWL029*. To locate these genes a blast has been performed revealing these genes in the *CWL029* genome. Green bars indicate the position of the oligos used, whereas black arrows show positions of putative promoters. Gene identification numbers, gene definitions and gene names can be consulted at **http://www.stdgen.lanl.gov**. and NCBI.

3.5.3. Transcriptional Interference led to the down-regulation of an alternative sigma factor in persistence

Especially intracellular pathogenic bacteria are exposed to a high evolutionary pressure that often leads to a very efficient arrangement of the genome. Publications have shown that overlapping genes may be a result of evolutionary pressure to minimize genome size (Sakharkar, K. R. *et al.*, 2004a). Therefore the comparison of frame shifting overlapping genes and especially convergent overlapping genes are of special interest to get a deeper understanding of the genome networks of *Cpn*. The expression profiles of the non-annoted genes of *CWL029* (CWL-NA-genes) described before were investigated with a focus on genes that are tandemly arranged with their neighbors. In most cases, these genes showed the same expression pattern as their neighbors and therefore it can be suggested that they belong to the same operon structure. Interestingly several CWL-NA-genes have a convergently oriented sequence overlap with neighboring genes. These include *Cp0063* in IGR 543, *Cp0396* in IGR 293 and *CP1040* in IGR 661. *Cp0063* and *Cp0396* are displayed in more detail in Figure 35.

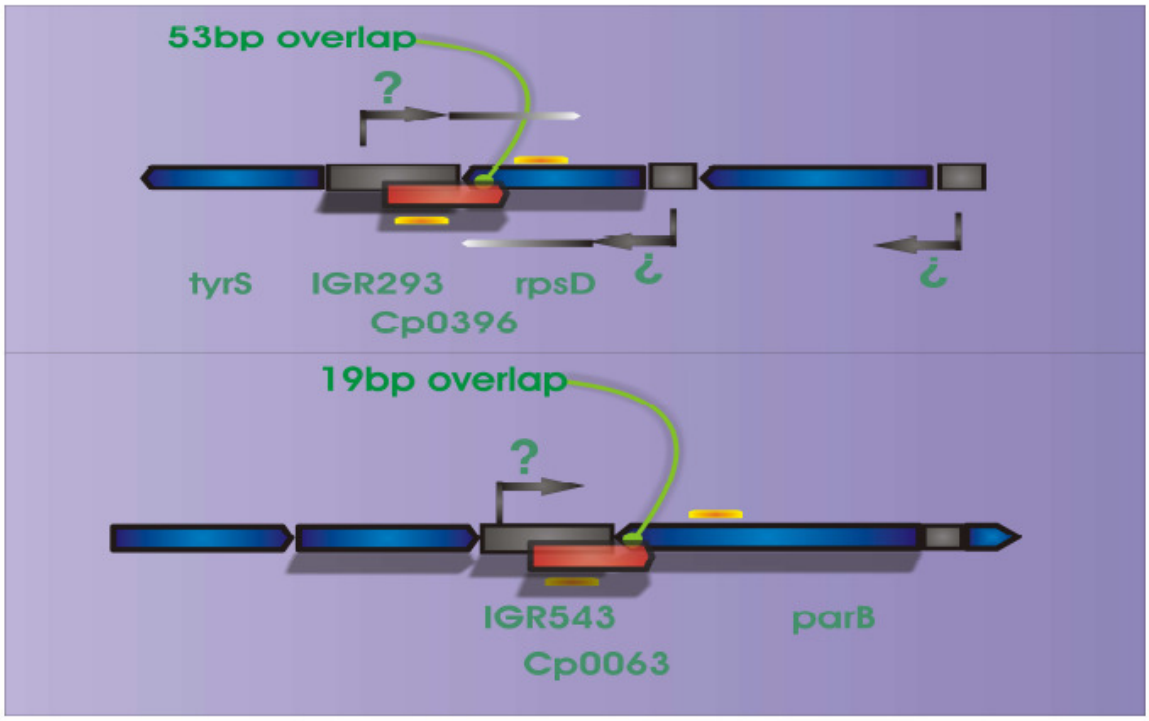


Figure 35 - **Schematic overview of the genome region neighboring IGR293 and IGR543.** *Cp0063* overlaps with *parB*, whereas *Cp0396* overlaps with *rpsD*, one of the alternative sigma factors. Genes in blue are *CWL029* genes, IGR are displayed in grey, whereas in red AR39 genes are displayed that have not been annotated in the *CWL029* genome.

Interestingly the associated genes which share a part of the sequence with these novel genes have key roles in the chlamydial developmental cycle. ParB is required for sporulation and normal chromosome segregation (Ogura, Y. *et al.*, 2003), whereas RpsD is an alternative sigma factor required for the transcription of 'Late' and 'Tardy' genes (Mathews, S. A. *et al.*, 1999a;Shen, L. *et al.*, 2004a). *Cpn0830* is a hypothetical gene with unknown function. The overlapping genes *Cp0063*, *Cp0396* and *CP1040* also are hypothetical genes with no homologue in *C. trachomatis* or other organisms and therefore are unique for *C. pneumoniae*. The expression profiles of these convergent overlapping gene pairs were investigated. For all gene pairs antagonistic expression profiles were observed, with *Cp0063* showing up-regulation at the beginning of the infection and *parB* reaching a maximum of expression at the end of the cycle (Figure 36). The same was the case for *Cp0396* which was up-regulated early during the cycle whereas *rpsD* was up-regulated late in the developmental cycle (Figure 37). Furthermore, in the persistent infection *rpsD* was down-regulated, whereas *Cp0396* was up-regulated (Figure 38 and Figure 39), also depicting an antagonistic expression. A comparison of the gene region of *Cp0396* and *rpsD* is presented in Figure 40.

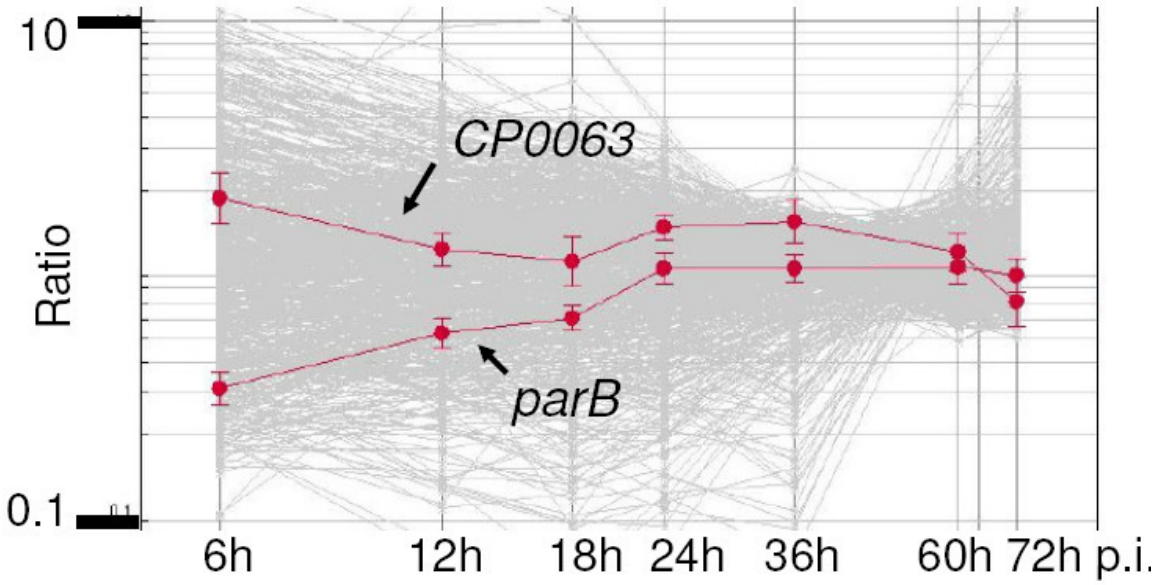


Figure 36 – **Trend plot for *Cp0063* and *parB* showing the expression during the acute infection.** X-axis represents the time (in hours) and the Y-Axis the expression level. The expression profiles of Cp0063 and parB are shown in blue, whereas all other genes are in grey. *Cp0063* and *parB* show an antagonistic expression profile in the developmental cycle.

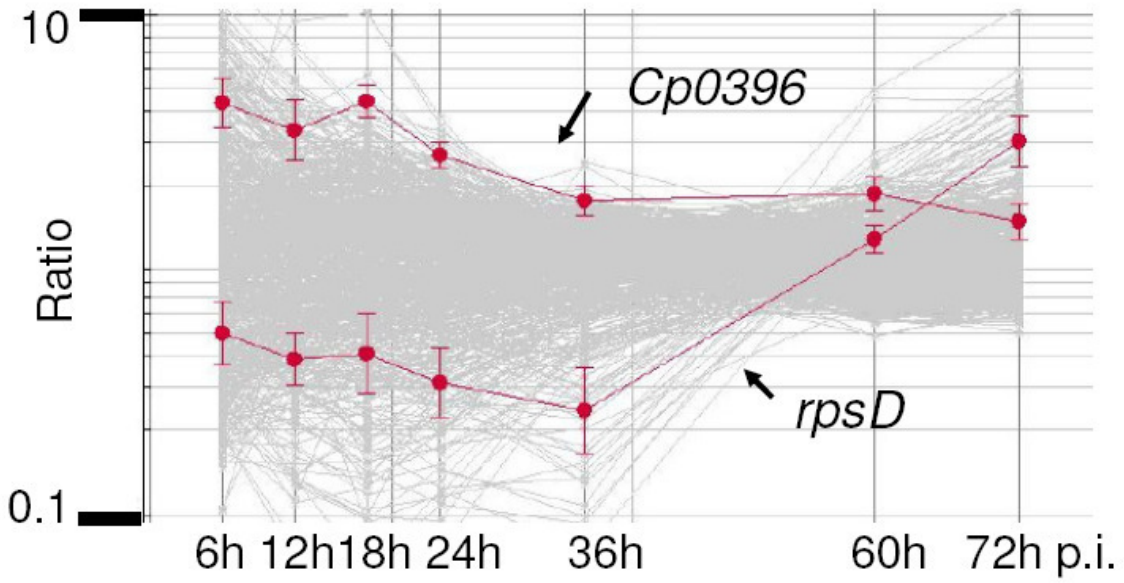


Figure 37 - **Trend plot for *Cp0396* and *rpsD* showing the expression during the acute infection.** X-axis represents the time (in hours) and the Y-Axis the expression level. *Cp0396* and *rpsD* show an antagonistic expression profile in the developmental cycle.

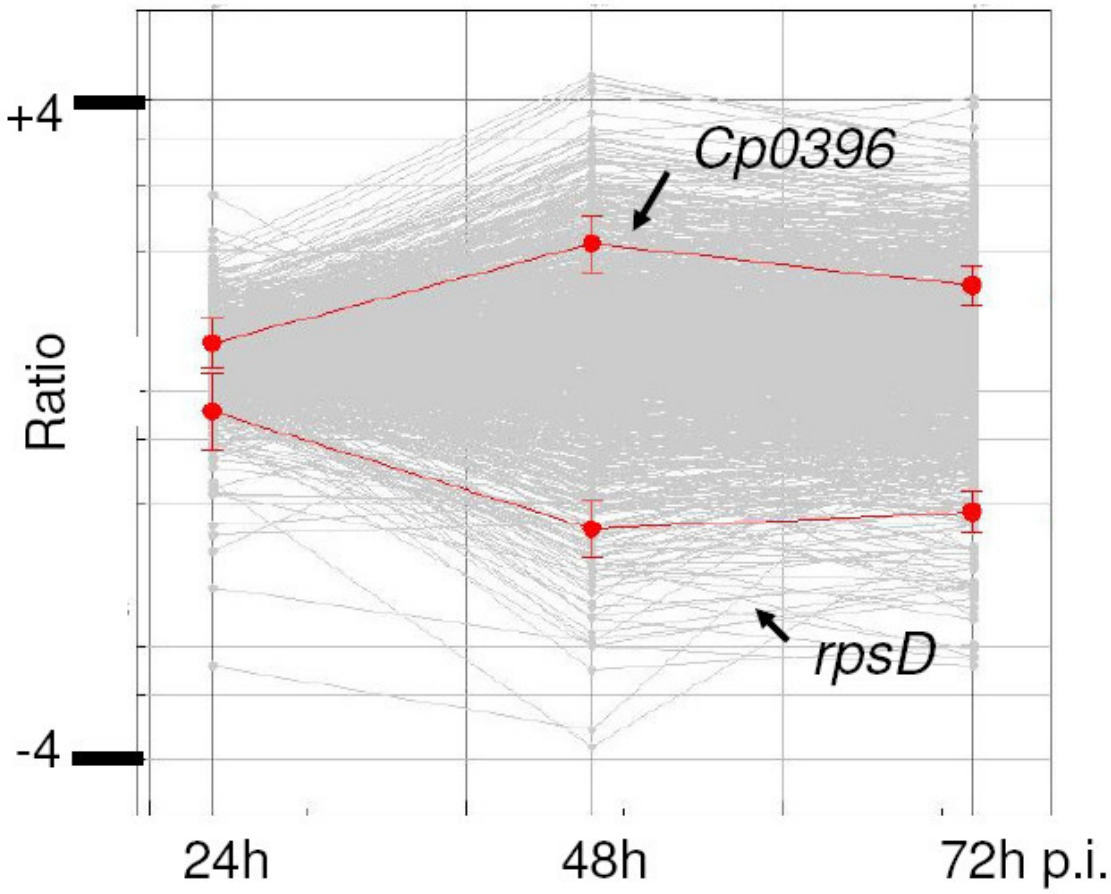


Figure 38 - **Trend plot for *Cp0396* and *rpsD* showing the expression during the persistent infection.** X-axis represents the time (in hours) and the Y-Axis the expression level. *Cp0396* and *rpsD* show an antagonistic expression profile in the persistent infection.

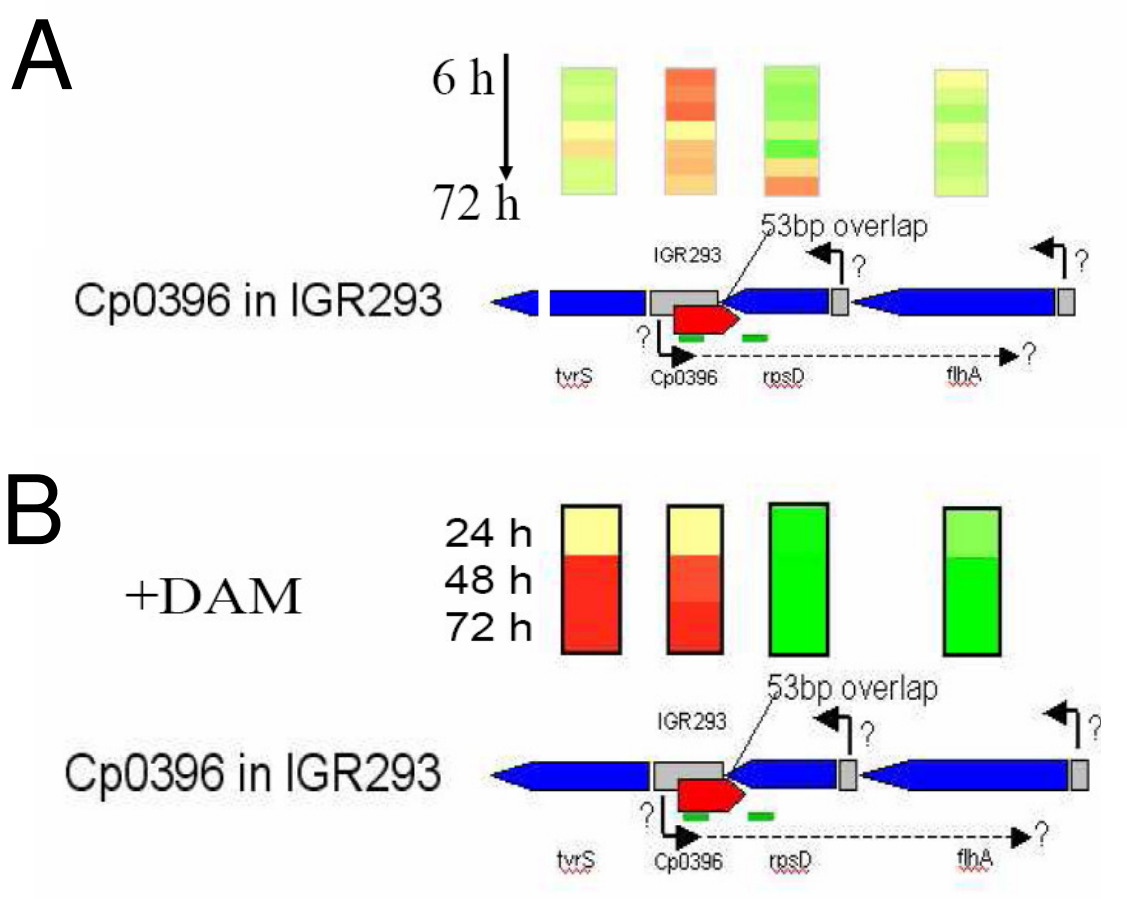


Figure 39 – **Heatmap display of the genomic region neighbouring IGR293** (A) for the acute and (B) for the persistent infection. In the acute infection *tyrS* and *flhA* show a constant profile, whereas *Cp0396* shows a declining and *rpsD* an increasing expression profile. In the persistent infection *tyrS* and *Cp0396* show an increased, whereas *rpsD* and *flhA* show an decreased expression profile compared to the equivalent time points of the acute infection.

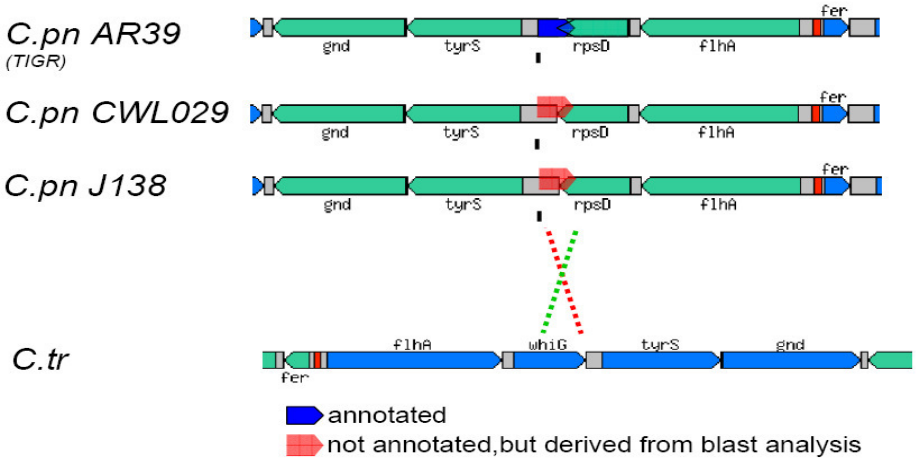


Figure 40 – **Genome region surrounding *rpsD*.** Schematic overview showing the genome region surrounding the described primer pair *Cp0396* (blue or red arrow, respectively) and *rpsD* for the sequenced *Cpn* strains and *Ctr.*

With the use of a prediction program (*http://www.fruitfly.org/seq_tools/promoter.html*) putative promoters for *Cp0396* and *rpsD* in the IGR293 and IGR294 were discovered. The sequences and the putative promoter elements are displayed in Figure 41 to Figure 43.

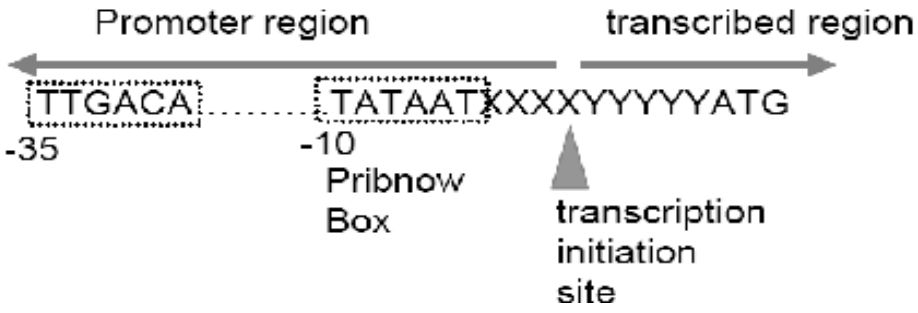


Figure 41 - **Model of a prokaroytic promoter region.**

Promoter prediction for CP0296 in IGR293

Start	*End*	*Score*	*Promoter Sequence*
79	*124*	*0.70*	*TAATTCCTTTGCTGAGATACCATAACTTCTATCTTCTCTA**A**AGATTAGGT*

IGR 293

GCTCTATCTTATCGATCCTAAAGCTTATATGCAAGGCACCACCTATCTAACAATAAAGAAAAGATT
*GTACGCTGAGAATAATTCCTTTGCTGAGATACCATAACTTC**TATCTT**CTCTA**A**AGATTAGGT*
CGCT **ATG**TCTACATCTCCAATTGGGGTTCCGTCGATG*CTAAACGCCGCAACTAGT*CTAAAT
GCCACAACTAGCAAGGCACCCCTTCCTACCTCTACCCTAGCCGAACGTATTAAAGAATGGCTGCC
CCGCATTCTTCTCTTGATTGTAGGAGCAAT

Figure 42 - **Promoter prediction for *CP0296* in the IGR 293**. One promoter has been found with a score value of 0.70. The transcription start and the putative Pribnow boxes are shown in a larger font.

Promoter predictions for rpsD in the reverse strand of IGR 294

Start	*End*	*Score*	*Promoter Sequence*
108	*63*	*0.66*	*TTAATTTAATCTTCTGTAAGCACTATTACTTTCGTATTTT**T**TTTGTTGGT*
79	*34*	*0.98*	*TTTCGTATTTTTTTTGTTGGTGTAAATATTGTTTAAAAAT**T**TTTTTTGAA*
68	*23*	*0.66*	*TTTTGTTGGTGTAAATATTGTTTAAAAATTTTTTTTGAA**T**TAATCTAATA*
59	*14*	*1.00*	*TGTAAATATTGTTTAAAAATTTTTTTTGAATTAATCTAAT**A**AATAACTAG*
46	*1*	*0.93*	*TAAAAATTTTTTTTGAATTAATCTAATAAATAACTAGAT**A**AAAAAAAATT*

IGR 294 (reversed)

*AAATTTTTTTT**T**ATCT**AGTTA**TTTATTAGATTAATTCAAAAAAAATTTTTAAACAATATTTACAC*
CAACAAAAAAAATACGAAAGTAATAGTGCTTACAGAAGATTAAATTAAA

Figure 43 - **Promoter prediction for *rpsD* in the IGR 294.** Several putative promoters have been found. The one chosen is highlighted in grey. The transcription start and the putative Pribnow boxes are shown in a larger font.

It has been shown that gene expression of one gene can influence the gene expression of neighboring genes (Chiaromonte, F. *et al.*, 2003b;Conte, C. *et al.*, 2002). This includes tandemly organized genes as well as divergently or convergently oriented genes (Prescott, E. M. *et al.*, 2002b;Callen, B. P. *et al.*, 2004b). In such cases transcription from one promoter can have a significant inhibitory effect on transcription from the other promoter, often with important regulatory consequences. This phenomenon, termed 'Transcriptional Interference', is poorly understood and it is not clear which factors determine the strength of interference and which mechanisms are most significant. This influence is thought to be mediated by an over read of one RNA polymerase into the other open reading frame. Several models of transcriptional interference have been suggested (Sneppen, K. *et al.*, 2005), including a strong (aggressive) and a sensitive (weak) promoter. The most popular models are the promoter occlusion, the sitting duck and the collision model (Figure 44).

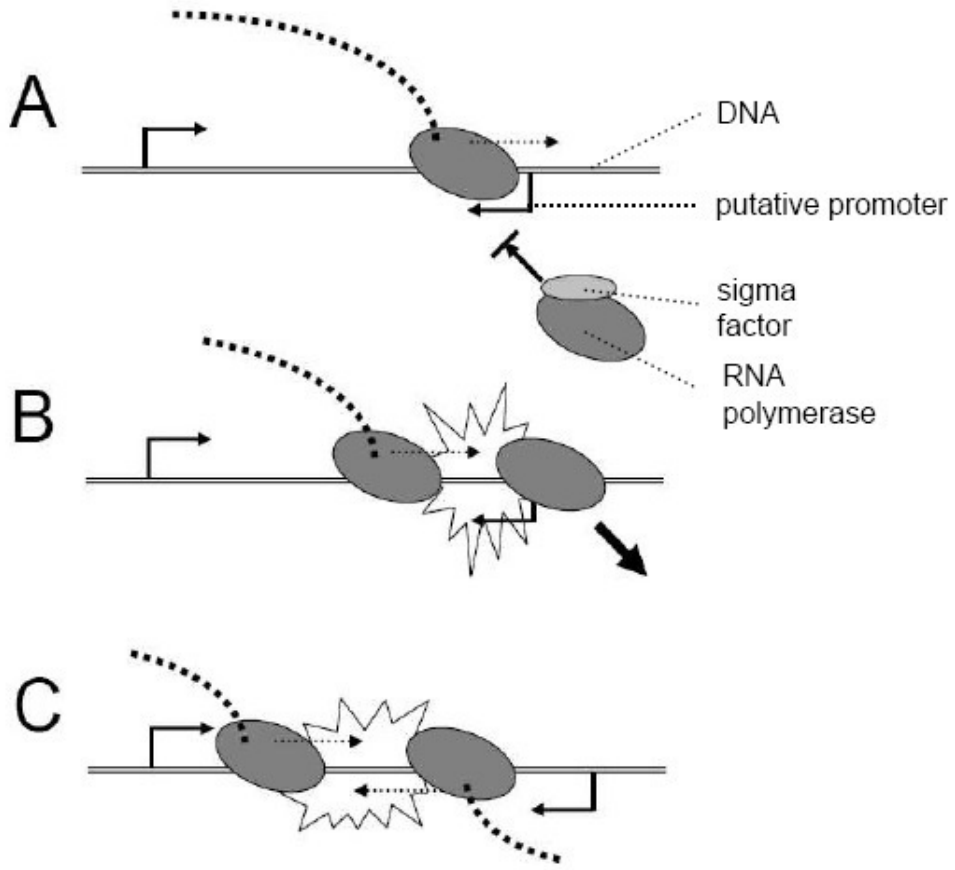


Figure 44 - **Models for transcriptional interference.** Several models have been discussed to result in 'Transcriptional Interference' for convergent organized promoters. This includes the occlusion model (A) in which the weak promoter is occupied because of a read through of the aggressive RNA polymerase, the sitting duck hypothesis (B) in which the RNA polymerase is not able to commence transcription because of a collision with the aggressive RNA polymerase and the collision model (C) where a collision of both RNA polymerases takes place during transcription.

In order to investigate if 'Transcriptional Interference' might be involved in the gene expression of the discovered overlapping genes a qRT-PCR experiment was performed as described in Figure 45. Specific primer pairs have been constructed to measure the effect of transcriptional interference. It can be expected that if transcriptional interference occurs the amount of PCR products decreases the longer the products are. The primer pairs are termed 0 for the IGR upstream of the putative *Cp0396* promoter and A to C for the shortest to the longest PCR product. The products from *Cp0396* are marked with a plus, whereas the products from *rpsD* are marked with a minus symbol.

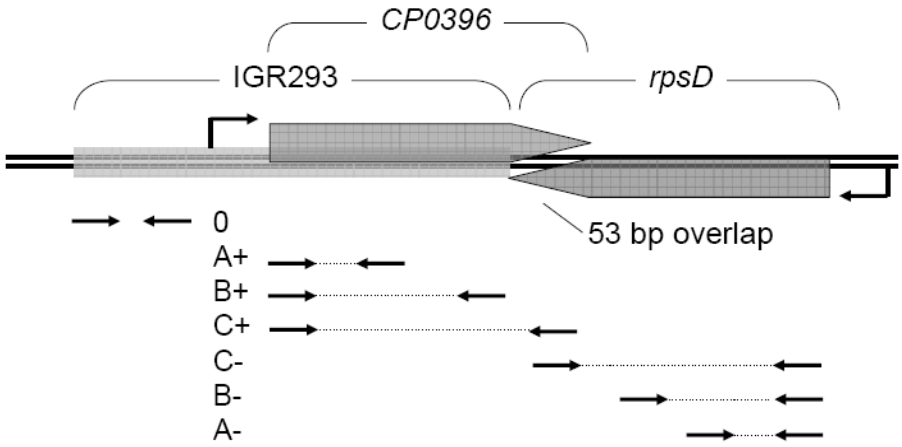


Figure 45 - **Primer design for *Cp0396* and *rpsD*.** To detect the involvement of transcriptional interference in the gene expression of *Cp0396* and *rpsD*, the following qRT-PCR experiment has been carried out. The following primer pairs have been designed: < 0 > for the IGR region upstream of the promoter, < A+ > to < C+ > for the detection of the increasing length of the *Cp0396* mRNA. The reverse primer for < C+ > anneals to the 53 bp overlapping region and < A- > to < C- > for the detection of the increasing length of *rpsD*.

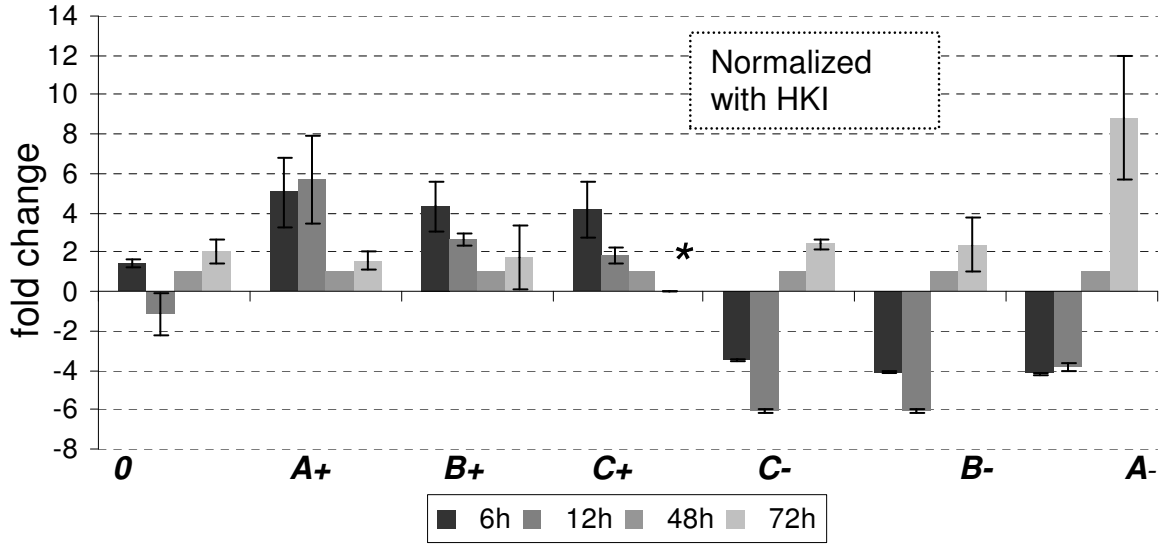


Figure 46 - **qRT-PCR results for *Cp0396* and *rpsD* in the acute infection**. The asterix indicates that no expression has been measured. Error bars indicate the standard error (SE) of three independent experiments. Expression values have been normalized using the HKI described before and with 48 h p.i. as a reference time point. The y-axis represents the fold change in comparison to 48 h p.i. the x-axis represents the used primer pairs.

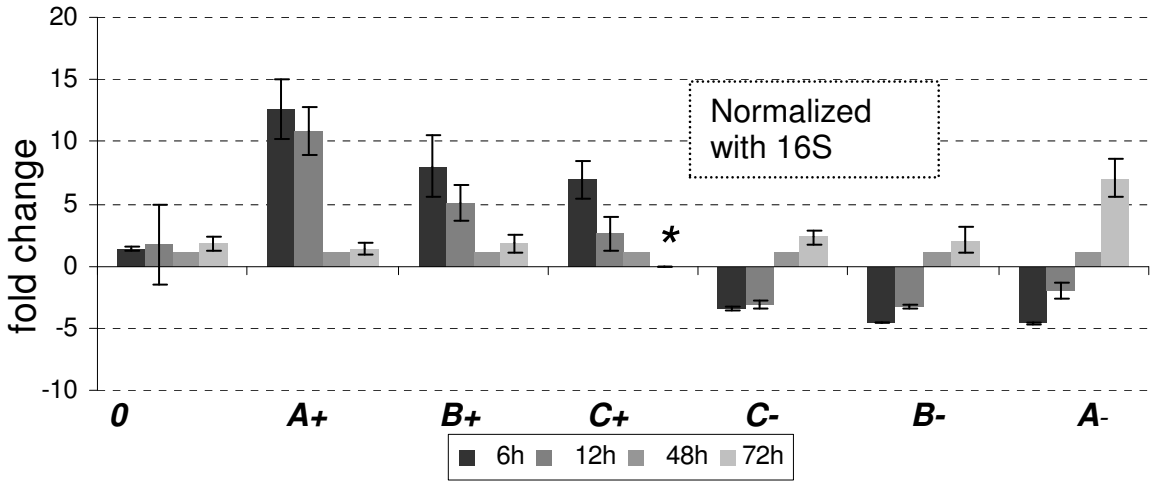


Figure 47 - **qRT-PCR results for *Cp0396* and *rpsD* in the acute infection.** The asterix indicates that no expression has been measured. Error bars indicate the standard error (SE) of three independent experiments. Expression values have been normalized using the 16S rRNA level and with 48 h p.i. as a reference time point. The y-axis represents the fold change in comparison to 48 h p.i. the x-axis represents the used primer pairs.

qRT-PCR gene expression measurement of *Cp0396* and *rpsD* (Figure 46, Figure 47) revealed the same expression pattern as indicated by the microarray data (Figure 39). Primer pair 0 did not show any expression at any of the measured time points, indicating that the predicted promoter position is correct. qRT-PCR with the primers A-plus, B-plus and C-plus being responsible for the amplification of *Cp0396* indicate an increased expression at 6 h and 12 h p.i, and a decreased expression at 48 h and 72 h p.i., respectively. Notably, at 72 h p.i. it was not possible to detect any expression via qRT-PCR for the full length transcript of *Cp0396* (primer pair C-plus), indicated with an asterix in Figure 46 and Figure 47. qRT-PCR measurement of *rpsD* expression showed a decreased expression at 6 h and 12 h p.i. followed by a 2-fold up-regulation of *rpsD* at 72 h post infection. The same expression values have been obtained when using 16S rRNA (Figure 47) instead of the HKI (Figure 46) for normalization, however with a higher standard error (SE) at 6 h p.i. Still both measurements are consistent with the microarray data obtained for the acute infection indicating an increased expression profile early in the cycle of development for *Cp0396,* whereas *rpsD* has an increased expression profile late in the cycle.

The mRNA expression level for *Cp0396* was unaltered in the persistent infection at 48 h p.i. compared to the same time point of the acute infection (Figure 48, Figure 48). At 72 h p.i. however, *Cp0396* expression increased 2-fold in contrast to the acute infection. Also in contrast to the acute infection at 72 h p.i., even for the full length *Cp0396* mRNA transcript (C+) expression was measured at 72 h p.i. in the persistent infection (Figure 46). For 48 h p.i. the *rpsD* expression was down-regulated about 2-fold, 4-fold and 6-fold when measured with the primer pair *A-minus*, *B-minus* and *C-minus* respectively. At 72 h p.i. qRT-PCR with *A-minus* primer pairs showed a 2-fold down-regulation, whereas with *B-minus* primer pairs a 1.3-fold and with *C-minus* primer pairs even a more than 2-fold up-regulation was measured. These results led to the following conclusions. First, instead of being down-regulated, *Cp0396* is up-regulated at late time points (72 h p.i.). Second, *Cp0396* produces full length mRNA transcripts for 72 h p.i. in the persistent infection, whereas for the acute infection no expression has been measured at this time point. Third, full length *rpsD* mRNA is down-regulated (*C-minus*), whereas the expression of middle size mRNA measured with primer pair *B-minus* is not altered. The expression of short mRNA measured with primer pair *A-minus* is even up-regulated more than 2-fold compared to 48 h p.i. in the acute infection. The data indicate that the *rpsD* promoter is active at 72 h p.i. in the persistent infection. However, the overlapping gene *Cp0396* is not down-regulated as it is at 72 h p.i. in the acute infection. This led most likely to ‘Transcriptional Interference’ between the two RNA polymerases. The position of collision seems to be on the side of *rpsD* and therefore no full length mRNA is produced, despite the *rpsD* promoter activation.

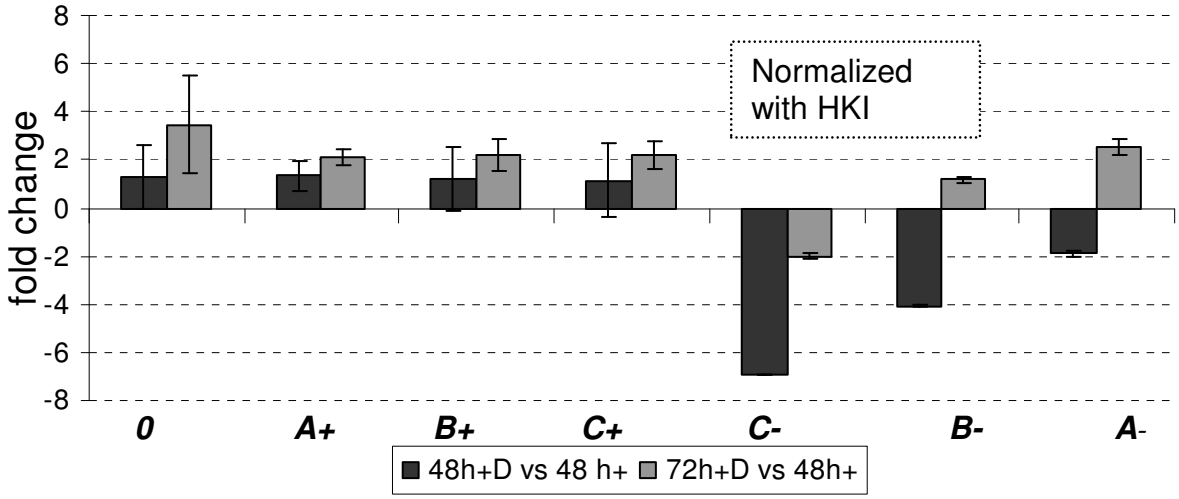


Figure 48 – **qRT-PCR results for *Cp0396* and *rpsD* in the persistent infection.** qRT-PCR results showing the expression during the persistent infection for 48 h p.i. and 72 h post infection. Expression values have been normalized using the HKI described before. Error bars indicate the standard error (SE). Values obtained for the persistent infection were normalized against 48 h p.i. of the acute infection. The y-axis represents the fold change in comparison to 48 h p.i. the x-axis represents the used primer pairs.

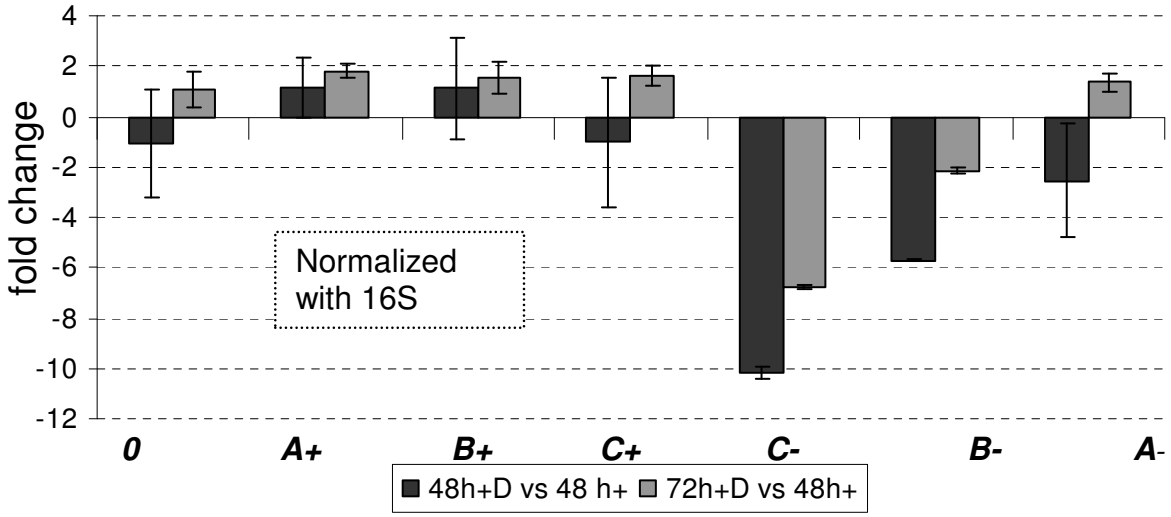


Figure 49 - **qRT-PCR results for *Cp0396* and *rpsD* in the persistent infection.** qRT-PCR results showing the expression during the persistent infection for 48 h p.i. and 72 h p.i. Expression values have been normalized using 16S rRNA. Error bars indicate the standard error (SE). Values obtained for the persistent infection were normalized against 48 h p.i. of the acute infection. The y-axis represents the fold change in comparison to 48 h p.i. the x-axis represents the used primer pairs.

3.5.4 Discussion

The data presented in this work revealed an impact of the gene orientation on gene expression of neighboring genes. At 6 h p.i. of the acute infection tandemly (>> or <<), divergently (<>) and convergently (><) oriented gene pairs showed mostly correlated expression. Only a minority of convergently oriented gene pairs revealed an antagonistic expression profile. However, at 72 h p.i. of the acute infection the picture changed. Whereas the majority of tandemly oriented gene pairs showed a correlated coexpression for divergently and convergently oriented gene pairs two groups were detected, one showing correlated expression and the other showing antagonistic expression. Moreover, at 72 h p.i. of the persistent infection most of the tandemly oriented gene pairs failed to show a correlated expression. Divergently and convergently oriented gene pairs could be grouped into two groups, one showing antagonistic expression, the other correlated expression. This indicated that gene expression of *Cpn* is linked to the orientation of the genes on the genome. The amount of gene pairs showing antagonistic or correlated expression changes throughout the developmental cycle. Moreover, in the persistent infection tandemly oriented gene pairs failed to show correlated expression, whereas for divergently and convergently oriented gene pairs a higher amount of genes showing antagonistic expression was discovered. The microarray analysis done for the *Cpn* strain *CWL029* revealed a number of open reading frames (ORFs) only predicted for the *Cpn* strains *AR39* and *J138*. The existence of these ORFs in the genome of *CWL029* was verified using BLAST. Interestingly several of these ORFs shared genome information with neighboring ORFs. Therefore the question arose, how overlapping genome information might influence the gene expression of these gene pairs. The *Cpn* genome is very condensed with 148 tandemly, 5 divergently and 44 convergently oriented gene pairs even sharing information on the genome and this question is therefore of general interest for *Cpn* gene expression. The expression of the gene pair *Cp0396 – rpsD* was analyzed in more detail using qRT-PCR. RpsD has been shown to be a responsible sigma factor for the expression of late and tardy genes (Shen, L. *et al.*, 2004d;Yu, H. H. *et al.*, 2003d). As it has been shown in this study that late and tardy genes are significantly connected to the iron mediated persistence, this indicated an important role of RpsD in the establishment of persistence. QRT-PCR data demonstrated that in the acute infection *Cp0396* and *rpsD* shared a perfect antagonistic expression profile with highest *Cp0396* expression at the beginning and highest *rpsD* expression at the end of the developmental cycle. However, in the persistent infection *Cp0396* was not down-regulated. Further, in persistence full length *rpsD* mRNA transcripts were down-regulated, whereas short length *rpsD* mRNA transcripts were up-regulated. The data demonstrated

that the *rpsD* promoter is activated at the end of the developmental cycle as well as in the persistent infection. Beside factor dependent transcriptional regulation (e.g. transcription factors) 'Transcriptional Interference' (TI) has been described to be involved in gene regulation. This mechanism has been observed in a variety of organisms, including the *temperate coliphage 186* (Callen, B. P. *et al.*, 2004a), *S. cerevisiae* (Martens, J. A. et al., 2004) and *E. coli* (Wang, P. et al., 1998) suggesting that TI co-evolved with factor dependent regulation. Several mechanisms have been suggested, with three of them acting in the initiation phase and two at the elongation stage of transcription (Gottesman, M. *et al.*, 1982;Liang, S. *et al.*, 1999;Prescott, E. M. *et al.*, 2002a). This led to the conclusion that the establishment of full length *rpsD* mRNA transcripts is hampered in *Cpn* persistence because its overlapping gene *Cp0396* fails to down-regulate. Most likely a collision takes place between the RNA polymerases acting on these convergently oriented genes. While other studies discussed factor dependent regulation for *rpsD* down-regulation (Gerard, H. C. *et al.*, 2002b;Douglas, A. L. *et al.*, 2000a) the data demonstrated that 'Transcriptional Interference' led to a down-regulation of full length *rpsD* transcripts in persistence. A decrease of full length mRNA consequently will also lead to a decrease of RpsD protein level as demonstrated by other studies (Wehrl, W. *et al.*, 2004e). Taken together, the microarray data and the qRT-PCR experiments performed in this study revealed the occurrence of 'Transcriptional Interference' between *Cp0396* and *rpsD* in persistence. Several other gene pairs might also be affected by 'Transcriptional Interference' in persistence. While most of the gene pairs sharing genome information are hypothetical genes, *parB* is of great importance. The *chromosome portioning protein B* (*parB*) is essential for chromosome segregation. Recent studies indicated that persistent Chlamydiae are inhibited in cell division and yet continue to accumulate chromosomes (Gerard, H. C. *et al.*, 2001a). ParB has been shown to be down-regulated in iron mediated persistence (Wehrl, W. *et al.*, 2004f). Therefore 'Transcriptional Interference' might be a key mechanism on the transcriptional level involved in the down-regulation of *rpsD* and *parB* full length mRNA consequently contributing to the establishment of the persistent infection. The finding that *Cp0396* is unique for *Cpn* led to the speculation that this mechanism might also contribute to the increased potential of *Cpn* to undergo persistent infections and to cause asymptomatic infections (Kuo, C. et al., 1998).

3.6 The host cell transcriptome during acute infection and iron mediated persistent infection of *C.pneumoniae*

Several studies have reported that Chlamydiae influence the host cell transcriptome (Hess, S. *et al.*, 2001;Hess, S. *et al.*, 2003;Mahony, J. B., 2002), whereas only a few genes are characterized to be differentially regulated in the chlamydial persistence (Peters, J. *et al.*, 2005). To investigate the impact of the *Cpn* infection and the iron depleted persistent infection on the host transcriptome microarray experiments using Agilent 8.1k arrays were performed. *Cpn* EB were ultrapurified as described and HEp-2 cells were infected with MOI 2 and harvested at 4 h p.i. and 24 h p.i. for the acute infection and the deferoxamine (DAM) induced iron persistence. RNA extracted from HEp-2 cells infected with UV-treated *Cpn* EB was included as a control (Figure 50).

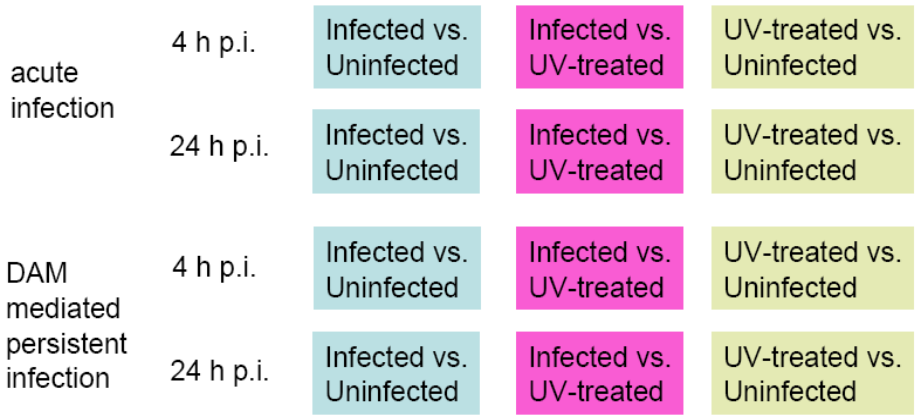


Figure 50 - **Experimental setup for the study of the impact of acute and persistent *Cpn* infection on the host cell transcriptome.** Experiments were done as two colour hybridizations with two duplicates.

RNA was extracted using the protocol for eukaryotic mRNA, amplified with a T7 promoter, labeled and hybridized onto the arrays. Data derived from the scanned slides were extracted using the Rosetta Resolver Biosoftware. Experiments were done in biological duplicates and with dye swap to ensure statistical relevance of the data. Using a 1.8-fold cut-off, the analysis derived 175 regulated genes for the non-infected (*NI*) vs. the infected (*I*), 275 genes for the UV-treated (*UV*) vs. *I* and 98 genes for the *NI* vs. *UV* hybridization at 4 h p.i. For the iron depletion persistence at 4 h p.i. 573 genes for the *NI* vs. *I*, 116 genes for the *UV* vs. *I* and 62 genes for the *NI* vs. *UV-treated* hybridization were derived. At 24 h

p.i. for the *NI* vs. *I* 169, for the *UV* vs. *I* 177 and for the *NI* vs. *UV-treated* hybridization 13 genes were derived for the acute infection, whereas for 24 h p.i. 242 genes for the *NI* vs. *I*, 143 genes for the *UV* vs. *I* and 157 genes for the *NI* vs. *UV* were obtained with the deferoxamine induced iron depletion persistence. To ensure the reliability of the derived data, a stringent data analysis was performed. Relevant genes were those being located in the overlap of the deived gene sets for the *NI* vs. *I* and *UV vs. I* hybridizations (Figure 51). Additionally, these genes were checked for correlated regulation (Figure 53) as it can be expected that reliable data show correlated expression for both the *NI vs. I* and *UV vs. I* hybridizations. This stringent data analysis led to 11 genes for the acute and 23 genes for the persistent infection at 4 h p.i. and 92 genes for the acute and 95 genes for the persistent infection at 24 h post infection (Figure 52). The same has been done to achieve the overlap for the gene sets triggered by alive and UV-treated and for the gene sets triggered only by UV-treated *Cpn*, with the only exception that for the latter analysis only genes showing anticorrelation were considered (Figure 53 to Figure 56).

3.6.1 Comparison of host gene sets triggered by viable Chlamydiae

No overlap exists between genes regulated by viable *Cpn* in the acute and persistent infection at 4 h p.i. (Figure 52). Also the comparison of genes regulated at 4 h p.i. and 24 h p.i. shows only a marginal overlap of two genes for the acute and one gene for the persistent infection. Comparing genes regulated in the acute and persistent infection at 24 h p.i. results in an overlap of 56 genes. Therefore, 9 genes are exclusively regulated in the acute infection and 22 genes in the persistence at 4 h p.i., whereas 33 genes are exclusively regulated in the acute infection and 35 genes in the persistent infection at 24 h p.i. (Figure 52). Taken together an increased influence on the host transcriptome could be seen for both the acute and persistent infection from 4 h p.i. to 24 h post infection. Interestingly at early stages of infection (4 h p.i.) the gene sets show no overlap and it therefore can be suggested that the acute and persistent infection triggers different host cell genes and pathways at this time point. At 24 h p.i. 56 genes could be detected in the overlap between acute and persistent infection, therefore suggesting a common influence on the host transcriptome. Nevertheless the genes exclusively triggered by acute and persistent *Cpn* infection are of great interest and therefore were analyzed at in much more greater detail.

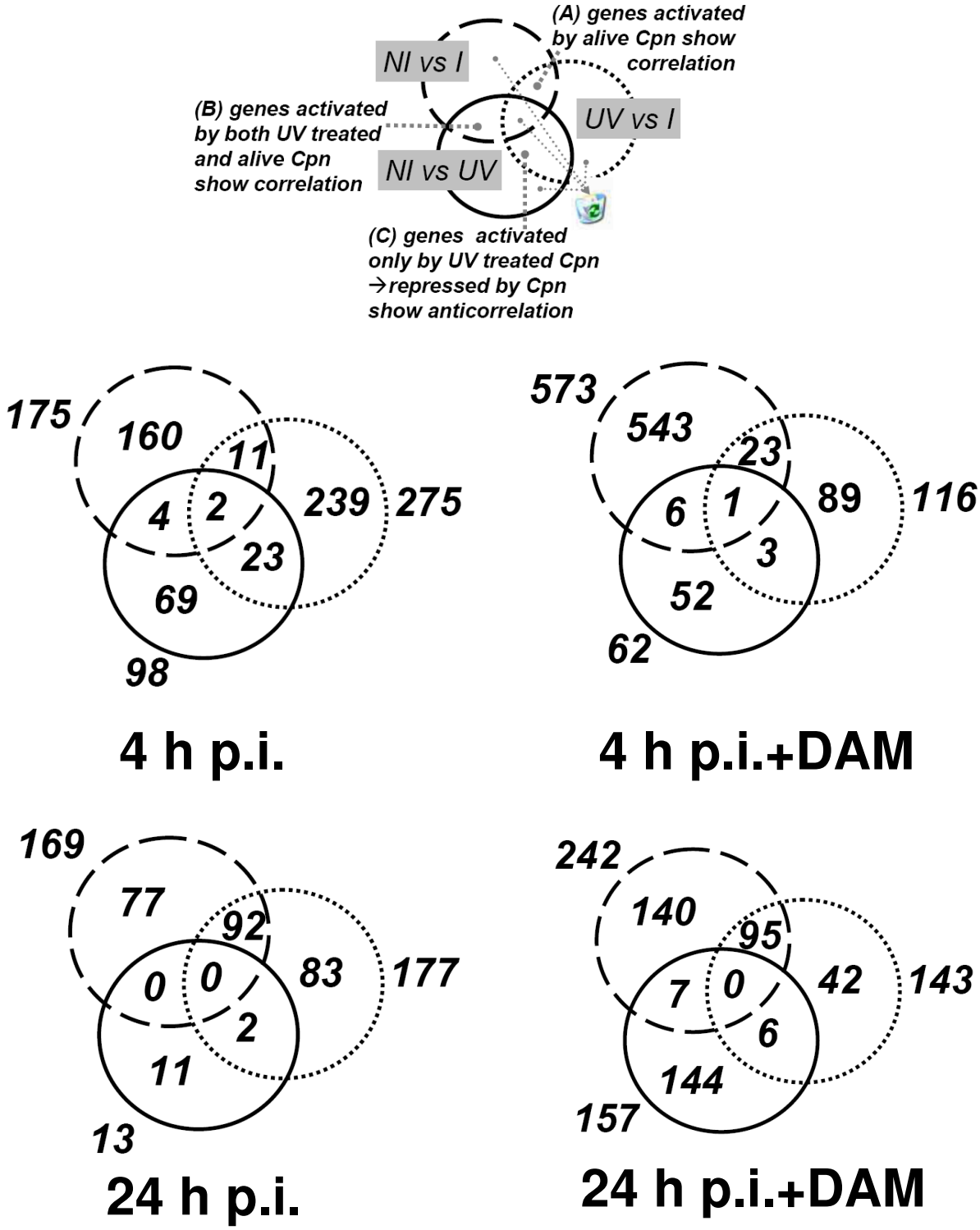


Figure 51 - **Gene sets derived from two colour hybridizations.** The data were compared for existing overlap between *NI* vs. *I* and *UV* vs. *I* for the detection of genes exclusively triggered by alive *Cpn*.; between *NI* vs. *I* and *NI* vs. *UV* to detect genes triggered by both alive and UV-treated *Cpn*, whereas the comparison of *UV* vs. *I* and *NI* vs. *I* led to a gene set triggered by UV-treated *Cpn*. Gene sets were derived for the acute infection at 4 h p.i., the persistent infection at 4 h p.i., the acute infection at 24 h p.i. and the persistent infection at 24 h p.i.

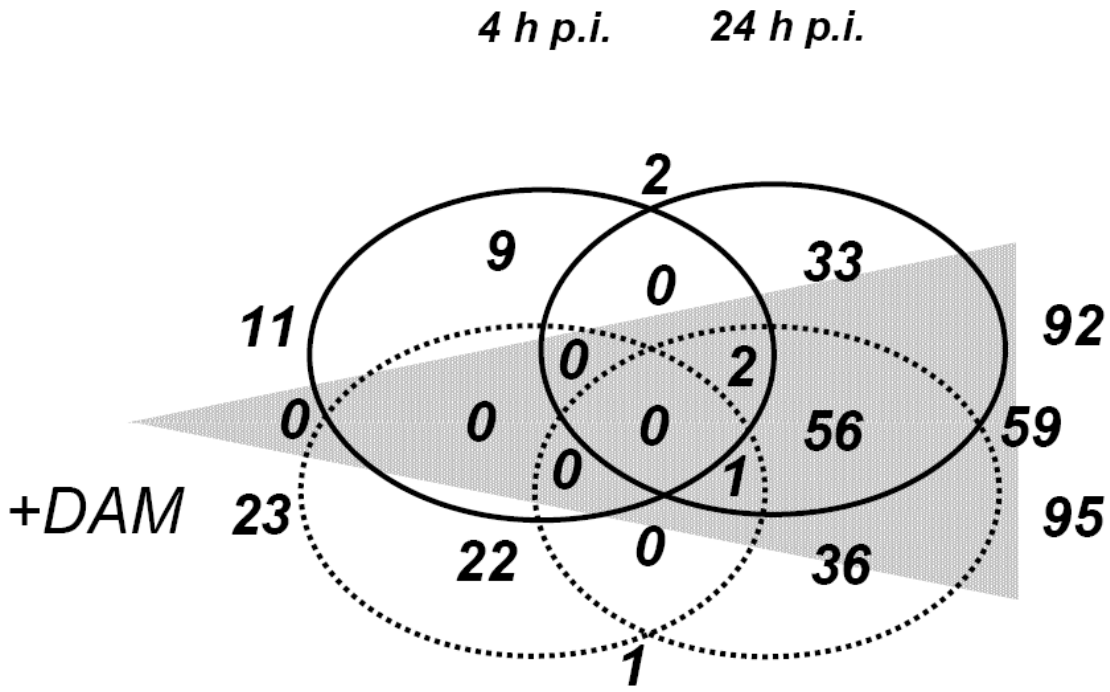


Figure 52 - **Comparison of the gene sets of host genes triggered exclusively by alive *Cpn*.** Significantly regulated genes for the acute infection (solid lines) and the persistent infection (dashed lines) at 4 h p.i. and 24 h p.i. are displayed. Genes present in different conditions or at different time points are displayed in the overlaps of the circles. Except for the overlap between acute and persistent infection at 24 h p.i. the overlap is only marginal .

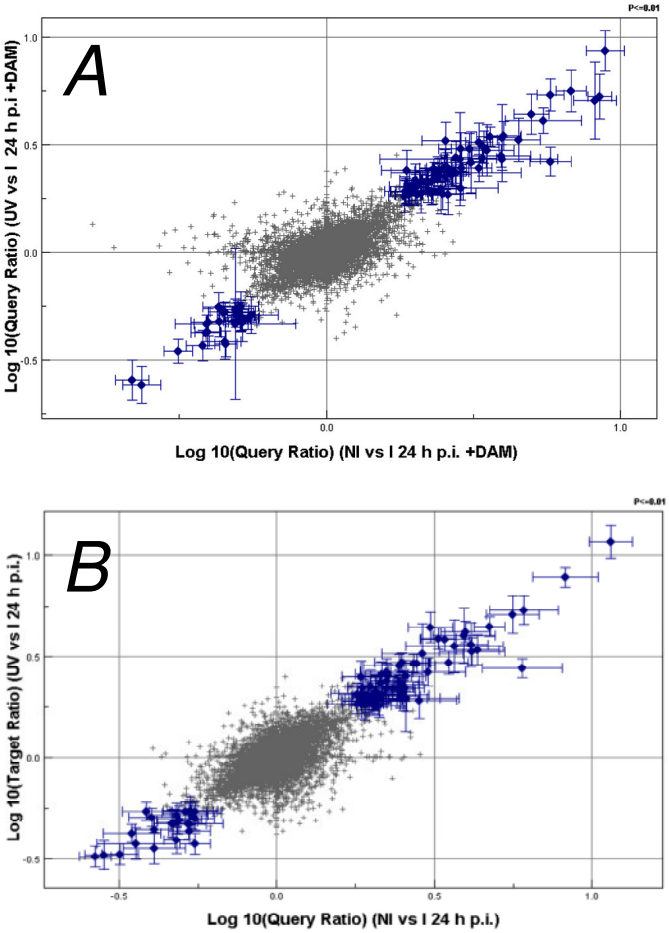


Figure 53 - **Data analysis for gene sets.** The data were commited to a very stringent data analysis. Only genes appearing in the overlap (see Figure 51) and additionally are showing a correlated expression were considered as trustworthy, whereas all others were handled with care. (A) shows the appearance of the 95 genes of the overlap of *NI* vs. *I* and *UV* vs. *I* hybridizations with all the genes showing correlated expression. (B) shows the same for the 92 genes of overlap between *NI* vs. *I* and *UV* vs. *I* hybridizations of the acute infection.

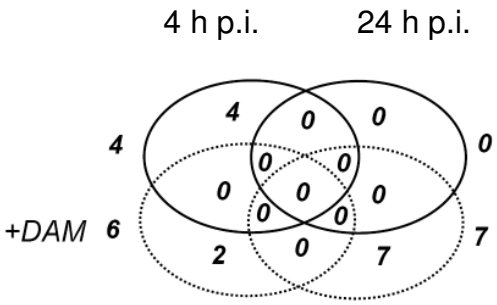


Figure 54 - **Comparison of host gene sets triggered by UV-treated and alive *Cpn*.** Significantly regulated genes for the acute infection (solid lines) and the persistent infection (dashed lines) at 4 h p.i. and 24 h p.i. are displayed. Genes present in different conditions or at different time points are displayed in the overlaps of the circles.

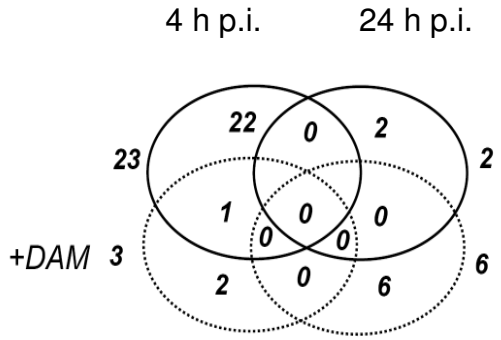


Figure 55 - **Comparison of host gene sets ttriggered exclusively by UV-treated *Cpn*.** Significantly regulated genes for the acute infection (solid lines) and the persistent infection (dashed lines) at 4 h p.i. and 24 h p.i. are displayed. Genes present in different conditions or at different time points are displayed in the overlaps of the circles.

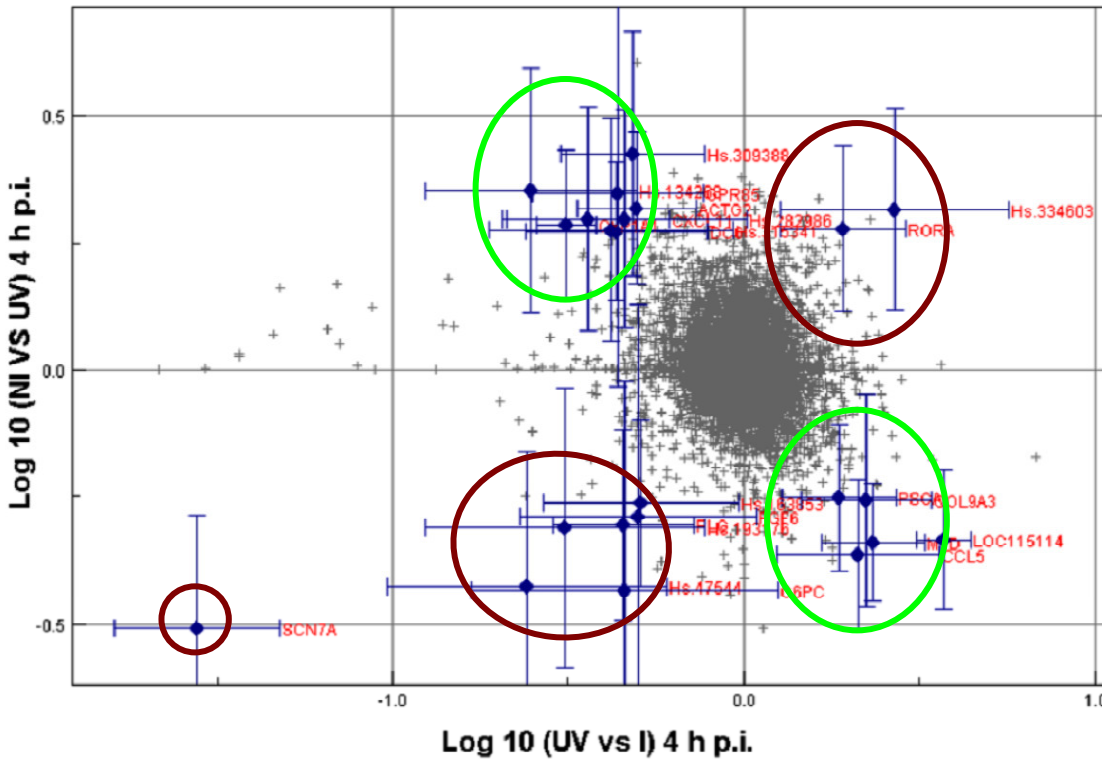


Figure 56 – **Compare plot for genes activated by UV treated *Cpn* at 4 h p.i. in the acute infection.** The X-axis shows UV vs. I hybridizations, the Y-axis NI vs. UV hybridizations. Data showing anticorrelation is marked with a green circle, data with correlation with a red circle. As shown in Figure 51E only anticorrelated genes are addressed.

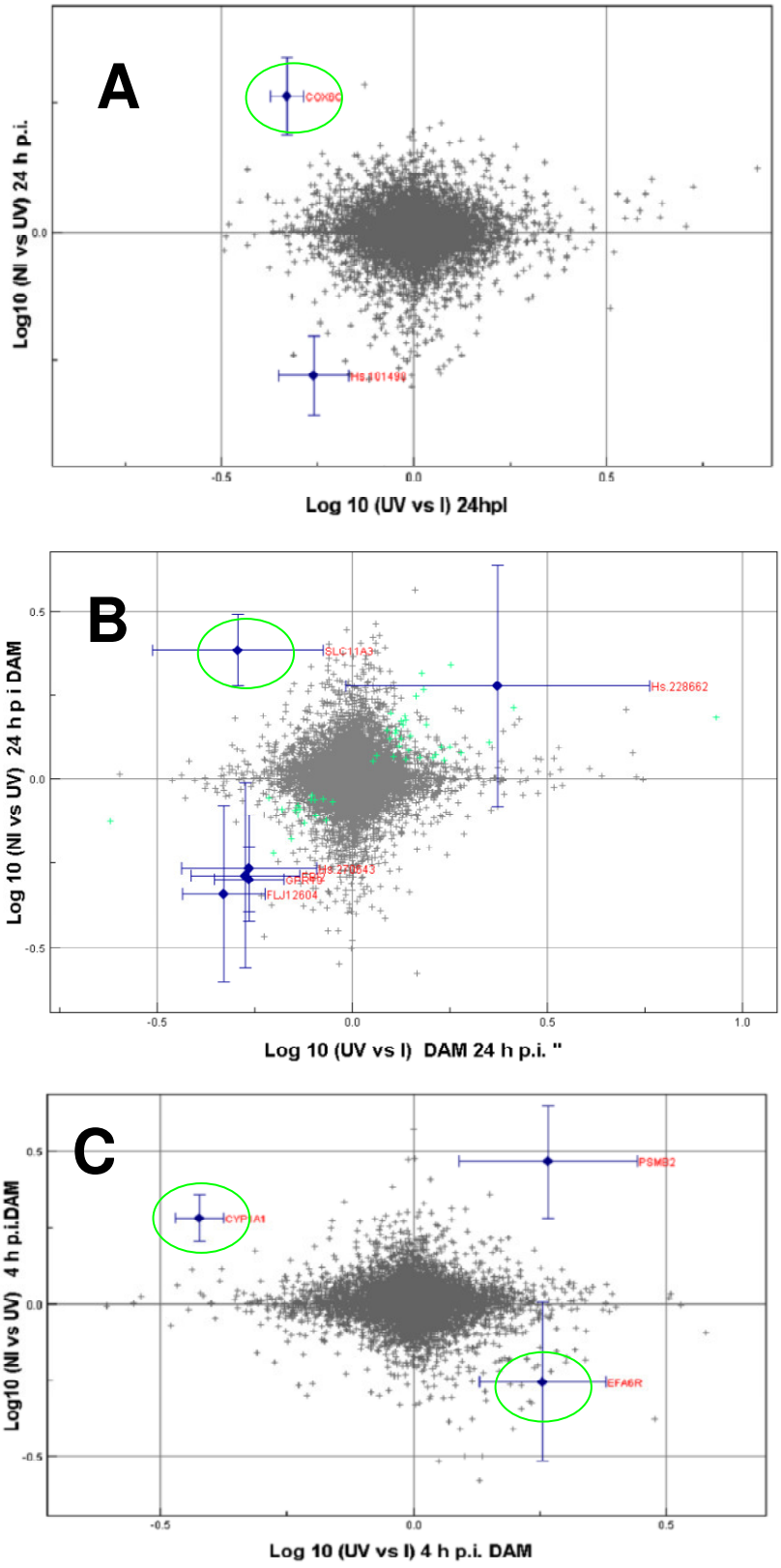


Figure 57 **– Compare plots for genes activated by UV treated *Cpn*.** (A) 24 h p.i. (B) 24 h p.i. DAM (C) 4 h p.i. DAM in the acute infection. The X-axis shows *UV* vs. *I* hybridizations, the Y-axis *NI* vs. *UV.* Data that shows anticorrelation is marked with a green circle. As shown in Figure 51 only anticorrelated genes are addressed.

3.6.2 *C.pneumoniae* induces an immediate host cell response at 4 h p.i.

To investigate the influence on the host transcriptome triggered by the acute and persistent infection, the derived gene lists were compared using the FatiGO (www.fatigo.org) software. FatiGO (Al Shahrour, F. *et al.*, 2004) is a web interface which carries out data mining using Gene Ontology for DNA microarray data. The comparison of genes regulated at 4 h p.i. showed a difference in several GO categories (Figure 58). Two categories, namely 'physiological processes' and 'response to a stimulus' showed a significant difference between the gene sets of the acute and persistent infection. 87.5% of the genes regulated in the acute infection, whereas only 9.09% of the persistent gene set could be grouped into the first category. The latter accounts for 75% of the acute gene set and only 18.18% of genes from the persistent infection. This included the down-regulation of *IFN-beta-1*, *crx*, *ltb*, *nrp2* and *pdyn* and the up-regulation of the genes *nr4a2*, *v-fos* and *cxcl-2* in the acute infection. For the persistent infection the down-regulation of *birc4*, *ovol-1*, *fgf11*, *tek*, *fzd5*, *tgfbr1* and the up-regulation of *cyr61*, *adamts1*, *sh2d1a* was observed. Interestingly, the performed stringent analysis showed no overlap between the 11 genes regulated in the acute and the 23 genes regulated during the persistent infection at 4 h p.i. (Figure 52).

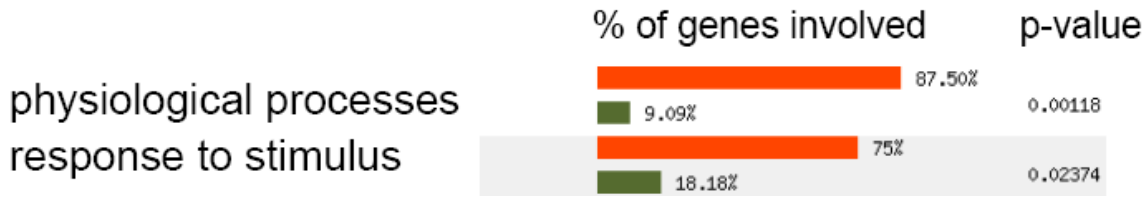


Figure 58 - **Comparison of differentially regulated genes between the acute and iron mediated persistent infection at 4 h p.i.** The first column shows the gene ontology term, the second the % of genes from the acute and persistent infection and the third the p-Value derived for this calculation showing if the difference is statistical significant or not. Red bars indicate the percentage for the acute, green for the persistent infection. The comparison has been done with FatiGO (**www.fatigo.org**) using gene ontology terms (note that to a certain gene, several GO terms can be connected, so that genes can show up in several groups).

Most of the genes differentially regulated at 4 h p.i. have been described to belong to an unspecific immediate response of the host cell due to stress (Supplementary-Table 4). As only few of these genes were also differentially regulated at 24 h p.i., this suggests that a switch from unspecific to more specific host cell answers to *Cpn* infection takes place in-between.

3.6.3 *C.pneumoniae* induces genes involved in cell death and cell proliferation at 24 h p.i. in the persistent infection

Beside three genes, the analysis of the host transcriptome at 24 h p.i. led to different gene sets for the acute and persistent infection when compared to 4 h p.i. 56 host genes were differentially regulated following the persistent as well as acute *Cpn* infection at 24 h p.i., whereas 33 genes were exclusively regulated in the acute infection, and 36 genes in the persistent infection (Figure 52)

Also the comparison of genes differentially regulated at 24 h p.i. with FatiGO led to different GO categories compared to 4 h p.i. (Supplementary – Table 5) (Figure 59 and Figure 60) . For two categories, namely 'cell death' and 'cell proliferation', a significant difference between the acute and persistent infection was investigated (Figure 61).

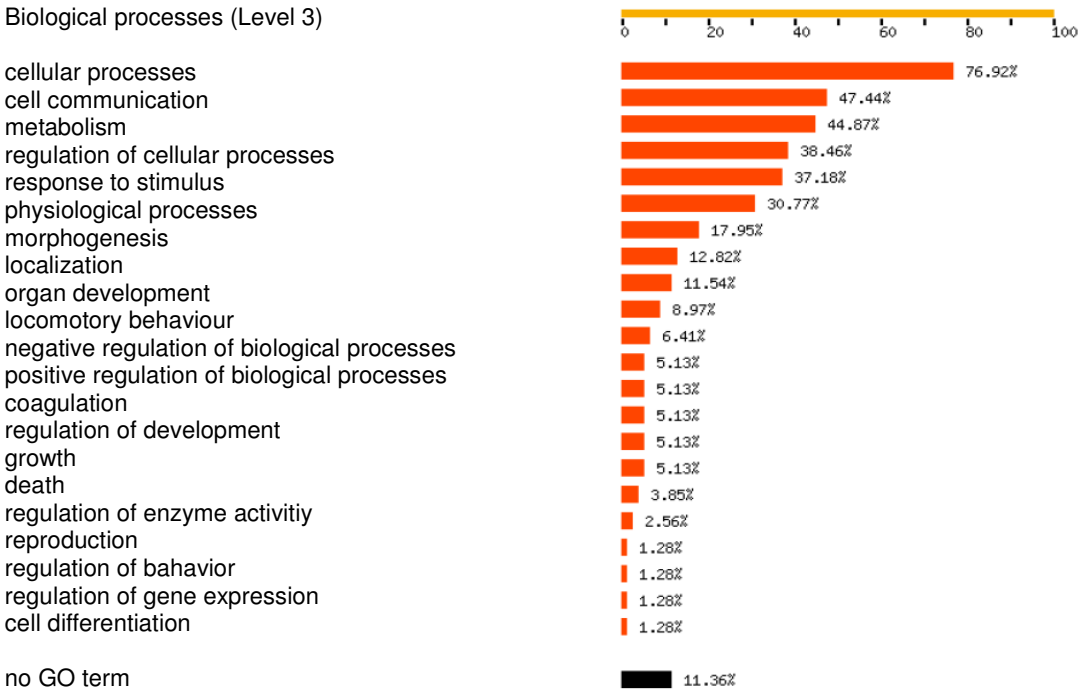


Figure 59 - **GO categories of the 92 differentially regulated genes for the acute infection at 24 h post infection.** The analysis has been done with FatiGO (**www.fatigo.org**) using gene ontology terms (note that to a certain gene, several GO terms can be connected, so that genes can show up in several groups).

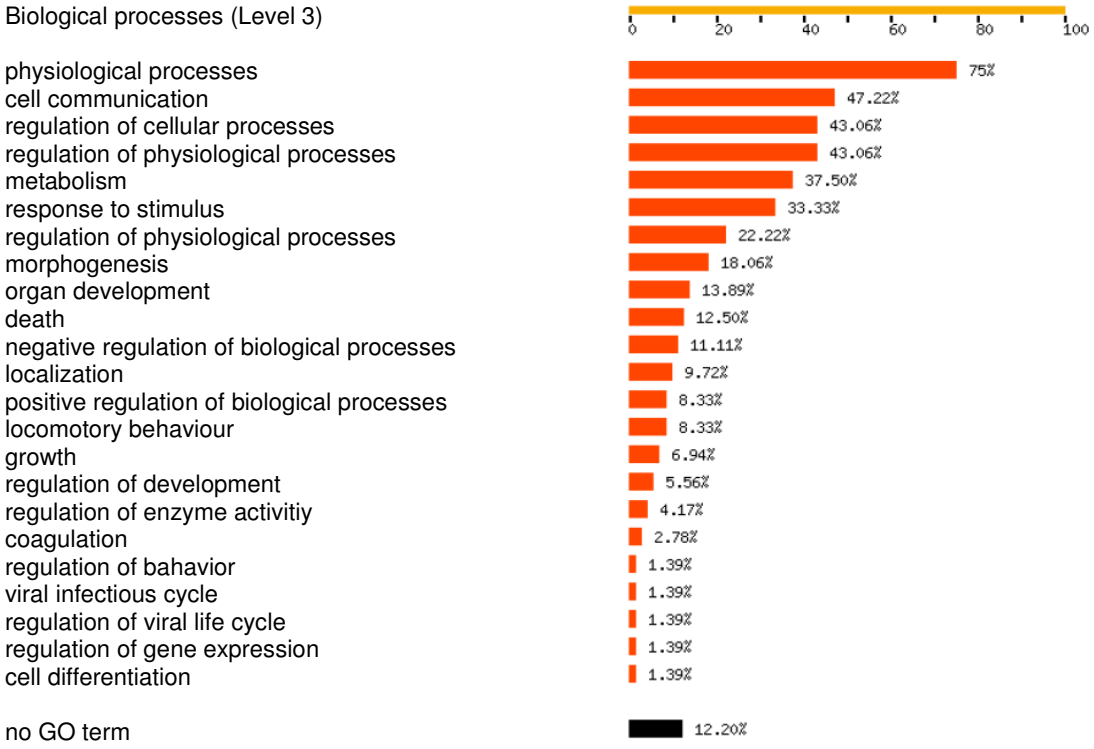


Figure 60 - **GO categories of the 95 differentially regulated genes for the persistent infection at 24 h post infection.** The analysis has been done with FatiGO (**www.fatigo.org**) using gene ontology terms (note that to a certain gene, several GO terms can be connected, so that genes can show up in

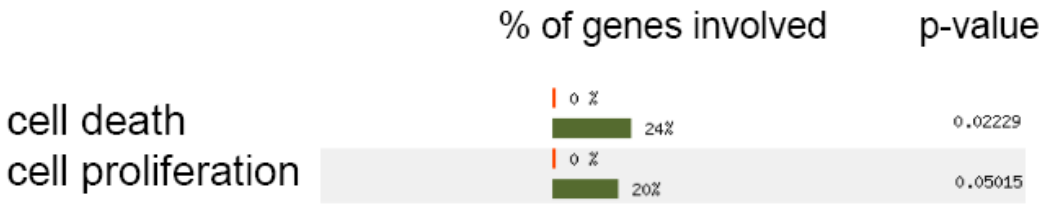


Figure 61 - Comparison of differentially regulated genes between the acute and iron mediated persistent infection at 24 h p.i. The first column shows the gene ontology term, the second the % of genes from the acute and persistent infection and the third the p-Value derived for this calculation showing if the difference is statistical significant or not. Red bars indicate the percentage for the acute, green for the persistent infection. The comparison has been done with FatiGO (**www.fatigo.org**) using gene ontology terms (note that to a certain gene, several GO terms can be connected, so that genes can show up in several groups).

3.6.4 *C.pneumoniae* infection triggers the expression of *relB*, involved in alternative pathways of the Rel/NF-kB transcription factor family

Chlamydiae are known to inhibit host cell apoptosis. However, the chlamydial antiapoptotic mechanism is still unclear. No NF-kappaB (NF-κB) activation was reported in *C. trachomatis* (*Ctr*) infected cells (Xiao, Y. *et al.*, 2005). In contrast, *Cpn* activates IKK/I-κB mediated signaling (Donath, B. *et al.*, 2002) and survival of *Cpn* infected cells is dependent on NF-ΚB activation and IΚ-Bα degradation (Wahl, C. *et al.*, 2001;Dechend, R. *et al.*, 1999a). Beside the NF-κB factors also RelA, RelB (v-rel reticuloendotheliosis viral oncogene homolog B), and c-Rel belong to the family of related transcription factors (Ghosh, S. *et al.*, 2002). While RelA is regulated through its cytosolic localization by Iκ-Bα, the regulation of RelB is mediated through transcriptional mechanisms and RelA alone is sufficient to mediate the stimuli-driven increase in *relB* transcription (Bren, G. D. *et al.*, 2001).

The microarray analysis revealed that *Cpn* infection triggers genes of the NF-κB factor family at 24 h p.i. in the acute as well as in the persistent infection. This comes along with an increased expression of *relB* and a moderate up-regulation of *nf-κB2* (Figure 62), both involved in alternative NF-κB pathways. Furthermore, *i-kappa-B-epsilon* (*i-κBε*) was up-regulated at 24 h p.i. (Figure 62). It was also possible to show that *Cpn* infection triggered up-regulation of the gene coding for the dual specificity mitogen-activated protein kinase kinase 3 (*mapk3* or *mkk3*) and the up-regulation of *tnf-α*, *death receptor 6* (*dr6*), *il-1β* and *il-8* in both the acute and persistent infection at 24 h p.i. (Figure 62).

Therefore it can be concluded, that two pathways are activated following *Cpn* infection: the NF-κB translocation-dependent pathway based on the degradation of Iκ-Bα and the NF-κB translocation-independent pathway involving the mitogen-activated protein kinase (MAPK) 3/6-p38 pathway (Watanabe, T. *et al.*, 2004). Both signaling pathways synergistically induce *tnf-α*, *il-1β*, and *il-8* expression.

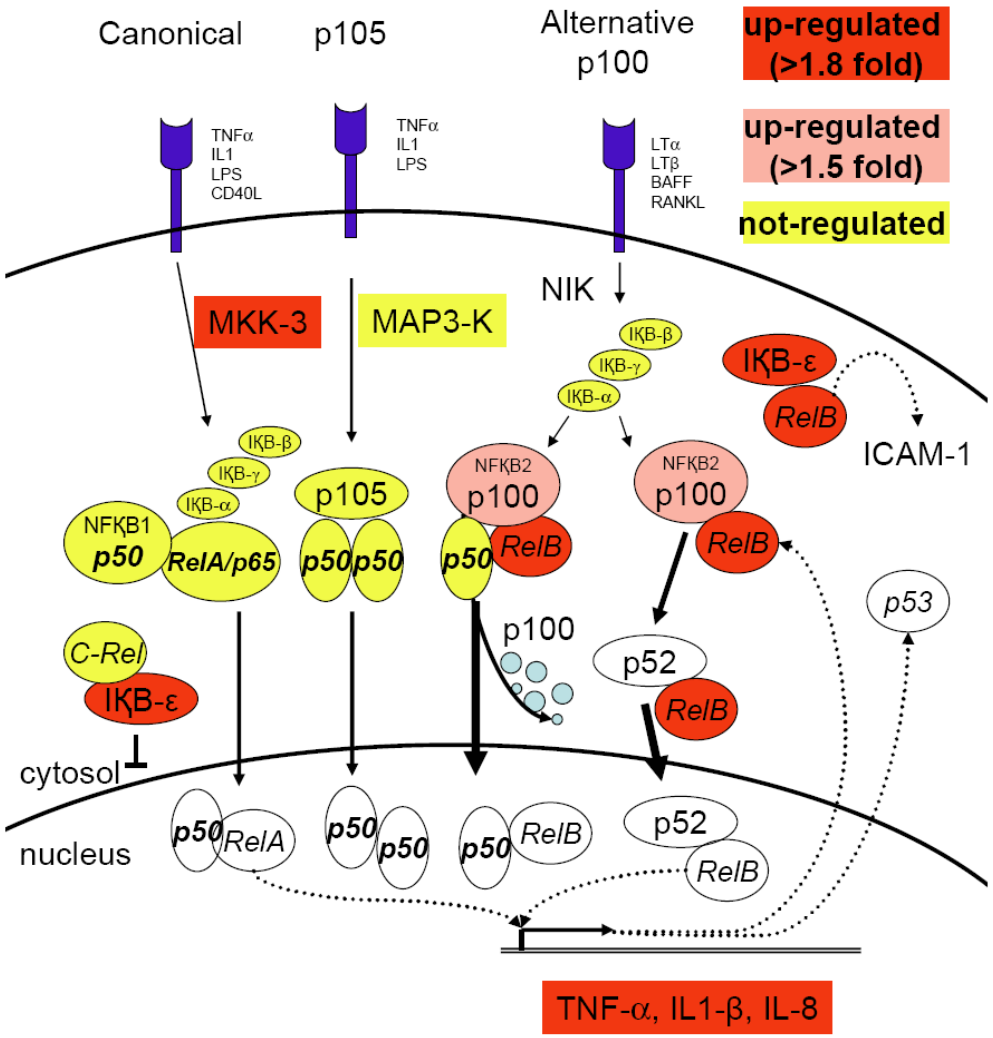


Figure 62 - **NF-κB pathways and regulation of genes involved in the *Cpn* mediated infection.** Red colors indicate up-regulation , green down-regulation and yellow no observed regulation on mRNA level. *RelB*, *iκB-ε*, *p100*, *mkk-3*, *tnf-α*, *il1-β* and *il-8* are up-regulated more than 1.8-fold, whereas *p100* is up-regulated more than 1.5-fold at 24 h p.i. RelB is involved in alternative NFκ-B pathways and *relB* transcription can be activated by nuclear RelA. The heterodimer RelB/IκB-ε is described to activate *ICAM-1* expression. Therefore it can be assumed that *Cpn* infection triggers alternative NFκ-B pathways.

Moreover, the transcriptome analysis revealed that *c-iap2* but not *c-iap1* is up-regulated following *Cpn* infection at 24 h p.i. in the acute and persistent infection. Also the up-regulation of *id-1* (inhibitor of DNA binding) exclusively in the acute infection is of interest. The heparin-binding, extracellular matrix-associated protein *cyr61* (*ccn1*) is up-regulated 3.8-fold in the acute and even 5.8-fold in the persistent infection. Taken together, this suggests that *Cpn* infection triggers host cell gene expression in a way that pathways are activated leading to cell survival, proliferation and anti-apoptosis at the same time.

3.6.5 *C.pneumoniae* infection triggers the expression of genes of the AP-1 transcription factor family

The activating protein transcription factors consist of dimers that belong to the Jun (c-Jun, v-Jun, JunB, JunD), Fos (c-Fos, v-Fos, FosB, Fra1, Fra2) and the related activating transcription factor (ATF2, ATF3/LRF1) subfamilies, however the exact subunit composition is influenced by the nature of the stimulus (JNK, ERK, etc.) that is activated.

The transcriptome data showed increased expression of *v-Jun* of 3.0-fold in the acute and 2.8-fold in the persistent infection at 24 h p.i. Interestingly at 4 h p.i. v-Jun was only up-regulated in the persistent but not in the acute infection. At 24 h p.i. also *v-fos* was up-regulated in the acute and persistent infection, whereas the remainder of the Jun and Fos transcription factors were not regulated at all. *Atf-2* was not differentially regulated following *Cpn* infection, whereas *atf-3* was only up-regulated in the acute but not in the persistent infection at 24 h post infection.

The data are consistent with a recent report showing that *Cpn* infection activates *v-Jun* (AP-1) (Miller, S. A. *et al.*, 2000). V-Jun, which overrides the mitogen dependence of S-phase entry is related to the NF-κB signaling pathways (Clark, W. *et al.*, 2000) and is implicated APK/JNK activated regulation of cell survival, apoptosis, and proliferation (Nishina, H. *et al.*, 2004;Kilbey, A. *et al.*, 1996). Moreover, the up-regulation of *atf-3* in the acute infection is an interesting finding as it has been shown recently that ATF-3 can counteract NF-κB-dependent anti-apoptosis (Hua, B. *et al.*, 2006) and also plays a role in the control of cell proliferation (Perez, S. *et al.*, 2001).

3.6.6 Genes involved in the host cell cycle are triggered following *C.pneumoniae* infection

In Chlamydiae infected cells multiple nuclei appear, indicating that infection inhibits host cell cytokinesis while allowing karyokinesis to proceed (Greene, W. *et al.*, 2003;Greene, W. *et al.*, 2004). This suggests that Chlamydiae may have evolved specific mechanisms for actively blocking host cell cytokinesis. Several genes involved in cell cycle control were triggered in the host cell following *Cpn* infection. The key factors regulated include *cyclin D1* and *cyclin G2*, with the former being up-regulated and the latter being down-regulated (Figure 63) at 24 h post infection. *Cyclin D1* was up-regulated 2.2-fold in the acute and even 3.1-fold in the persistent infection, whereas c*yclin G2* was about 2-fold down-regulated in both conditions. Moreover, the expression data also revealed that *p57-Kip* (Cyclin-dependent kinase inhibitor p57), $p15^{INK4b}$ and $p18^{INK4c}$ were down-regulated at 24 h following *Cpn* infection (Figure 63). While *p57-Kip* was down-regulated 2.8-fold in the acute and 3.8-fold in the persistent infection , $p15^{INK4b}$ and $p18^{INK4c}$ were down-regulated more than 2-fold in the persistent infection but only little affected in the acute infection. Further several p53 downstream targets genes were differentially regulated, including *gadd45a* and several genes coding for DUSP phosphatases. However, a differential expression of *p53* at a transcriptional level was not observed. Interestingly, *gadd45A* was only up-regulated at 24 h p.i. in the persistent but not in the acute infection. Also other genes coding for proteins influencing cell cycle progression have been found to be triggered by *Cpn* infection. This includes the down-regulation of *brd3***,** negatively regulating cell proliferation (Ishii, H. *et al.*, 2005b;Ishii, H. *et al.*, 2005a) and *lamin B1*, a component of the nuclear envelope (Moir, R. D. *et al.*, 2000a). Entry of quiescent cells into the cell cycle is driven by Cyclin D-dependent kinases Cdk-4 and Cdk-6 which are negatively regulated by the cell cycle inhibitors P15 and P18 (Sherr, C. J. *et al.*, 1995;Noh, S. J. *et al.*, 1999). Also the cyclin-dependent kinase inhibitor P57-Kip is a negative regulator of the cell cycle (Noura, S. *et al.*, 2001;Zhang, P. *et al.*, 1997). Whereas *cyclin D1, cyclin G2* and $p75^{Kip2}$ are differentially regulated in both the acute and persistent infection, other mediators of cell cycle progression, like *p15*, *p18* or *gadd45A* are affected to a lesser extent in the acute than in the persistent infection. Therefore it can be speculated that in the persistent infection additional mechanisms influence the host cell cycle. Lamin B1 e.g. is required for nuclear integrity (Vergnes, L. *et al.*, 2004) and it has been shown that during the anaphase-telophase transition, Lamin B1 concentrates at the surface of the chromosomes (Moir, R. D. *et al.*, 2000b). Taken together, these findings indicate that *Cpn* infection has a major impact on cell cycle regulation of the host cell.

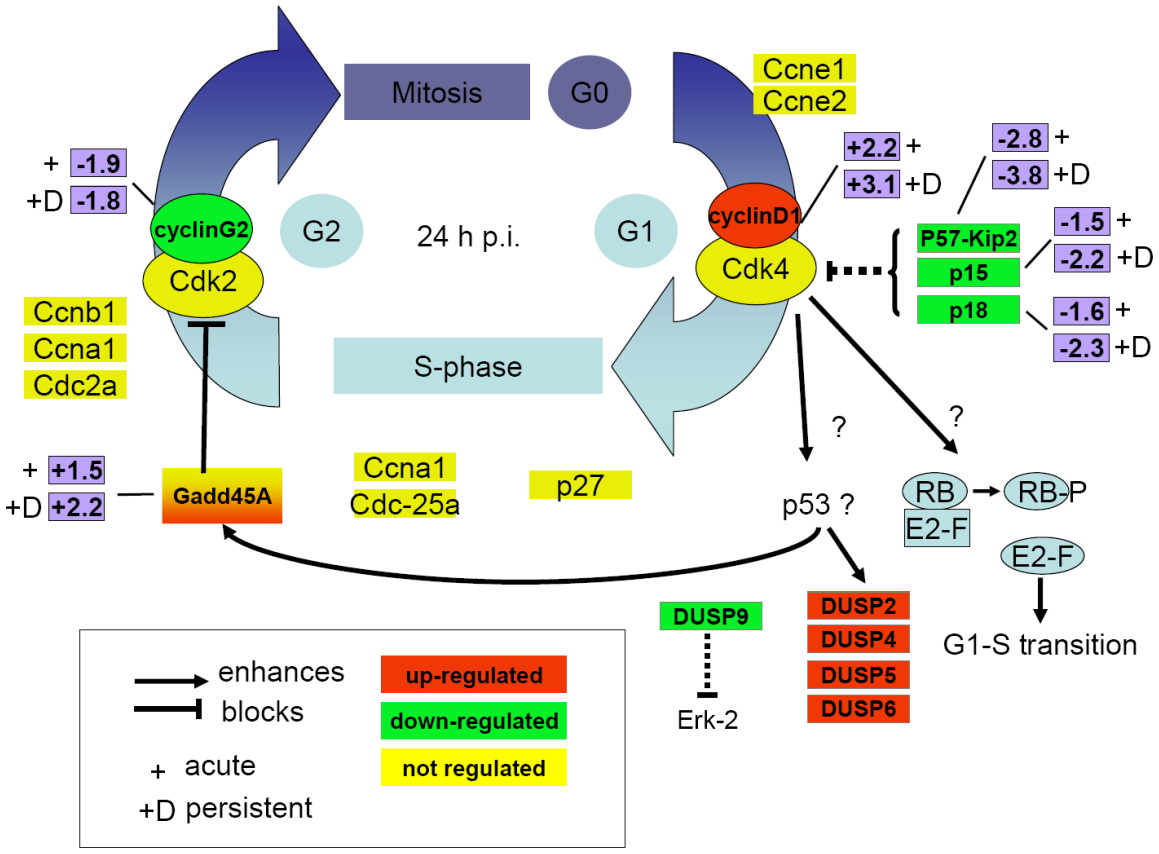


Figure 63 – **Influence of *Cpn* infection on genes involved in the host cell cycle at 24 h post infection.** Red colors indicate up-regulation, green down-regulation and yellow no observed regulation. For some genes the regulation is indicated with + for the acute and +D for the persistent infection. *Cyclin D1* is up-regulated, whereas *cyclin G2, p57, p15* and *p18* are down-regulated. Both, *cyclin D1* up-regulation and *cyclin G2, p57, p15* and *p18* down-regulation are connected to G1-S transistion. Moreover, the CyclinD1/Cdk4 complex can activate *p53* and mediate G1-S transistion. Downstream target genes of P53 were up-regulated including genes for DUSP kinases and *gadd45A*, with the latter being described to block Cdk2 function. Interestingly *p15*, *p18* and *gadd45A* were only differentially expressed in the persistent but not in the acute infection.

3.6.7 P53 might be a mediator of transcriptional regulation in *C.pneumoniae* infected host cells

This study showed that *gadd45a* was 2-fold up-regulated, whereas *puma* was 2-fold down-regulated in the persistent infection. As both genes are downstream genes of the tumor suppressor P53 (Yu, J. *et al.*, 2005a) those results are contradictious. PUMA can be regulated at the transcriptional level by the activation of transcription factor P53 (Oda, E. *et al.*, 2000;Yu, J. *et al.*, 2001). Therefore, the observed down-regulation of PUMA in the persistent *Cpn* infection also indicates an involvement of P53. Most of the proapoptotic effect of p53 and its homologous is related to the transcriptional up-regulation of several pro-apoptotic genes coding for proteins like Bax, Apaf-1, Fas, Killer/DR5, Noxa, P53, AIP and PUMA (Vousden, K. H., 2002). Consequently the downregulation of *puma* in *Cpn* infected cells has been described as an anti-apoptitic effect (Fischer, S. F. *et al.*, 2004b;Fischer, S. F. *et al.*, 2004a). Also *dusp2* has been shown to be up-regulated in this study. It is a downstream target of P53 also causing cell cycle arrest (Yin, Y. *et al.*, 2003). Therefore the question arises how P53 might be involved in the regulation of these downstream targets. Several mechanisms of P53 deactivation have been described to function on a post-transcriptional level. Expression of *mdm2* is activated by P53 and accumulation of the Mdm2 protein, regulating the stability of the P53 protein by ubiquitination has been observed (Alarcon-Vargas, D. *et al.*, 2002). However, this study showed that *mdm2* is not affected on a transcriptional level in the *Cpn* infection. Also *parc*, leading to an inactivation of P53 (Nikolaev, A. Y. *et al.*, 2003) was not regulated. The regulation of P53 is complex. A recent publication showed that P53 can not only up-regulate *puma* to mediate apoptosis but also *slug*. Slug represses the transcription of *puma*, thereby preventing apoptosis (Wu, W. S. *et al.*, 2005b). The activation of gadd45A expression has also been described to be possible in a P53 independent manner (Yoshida, T. *et al.*, 2005).
The *p53 dependent modulator of apoptosis* (*puma*) was 2-fold down-regulated in the persistence and 1.6-fold down-regulated in the acute infection. This is consistent with the anti-apoptotic effect of *Cpn,* but on the other hand *puma* is a P53 downstream target gene (Yu, J. *et al.*, 2005b). As the transcriptome data suggest an activation of the P53 pathway, *puma* down-regulation might even indicate a fine tuning of these important pathway. Indeed it has been shown that p53 activation and *puma* down-regulation can be mediated by the up-regulation of other p53 downstream target genes (Wu, W. S. *et al.*, 2005a).

3.6.8 *C. pneumoniae* infection triggers genes involved in membrane trafficking and transport of metabolism

Cpn infection alters the expression of host genes playing an essential role in membrane trafficking and in the structural and functional organization of a cell: *rab40B* was about 2-fold down-regulated at 24 h p.i., following acute and persistent *Cpn* infection. Rab40B is a homolgue of Rab40c, that is localized in the perinuclear recycling compartment, suggesting its involvement in endocytic events such as receptor recycling (Rodriguez-Gabin, A. G. *et al.*, 2004). Further, *rab26* was 2.5-fold down-regulated 24 h p.i., following acute or persistent infection. Both Rab40B and Rab26 are GTP binding proteins and effectors of the Ras-Rho/GTPase signaling pathway (Jordens, I. *et al.*, 2005). In contrast *rin1* was up-regulated 2-fold in the acute and 1.6-fold in the persistent infection 24 h post infection. Rin1 is a multifunctional protein containing several domains, including Ras binding and Rab5 GEF domains (Hunker, C. M. *et al.*, 2006). Other genes coding for effectors as *rhoB* or modulators, like *rgs14*, *rin3* or *pde6B* of the Ras-Rho/GTPase pathways were not differentially expressed. Beside membrane trafficking, several genes involved in metabolic pathways and metabolism transport were also affected. This included *pld1* which was down-regulated more than 2-fold at 24 h p.i., following acute and persistent infection. PLD1 is involved in mTOR signaling and cell size control (Fang, Y. *et al.*, 2003).

The persistent infection showed genes being differentially regulated compared to the acute infection. This involved *aquaporin-1* (*aqp-1*) and *aquaporin-7* (*aqp-7*) (Figure 64). While *aqp-1* was down-regulated 1.7-fold in the acute and about 2-fold in the persistent infection, *aqp-7* was not affected in the acute but down-regulated 2.2-fold in the persistent infection at 24 h post infection. Aquaporins are involved in volume regulation and often additional other mechanisms, such as solute transporters, are involved (Verkman, A. S., 2005). Following *Cpn* infection several genes coding for solute transporters were differentially regulated. This included *slc12A2* (up-regulated 2.6-fold in the acute and 1.7-fold in the persistent infection at 24 h p.i.), *slc16A3* (up-regulated 1.8-fold in the acute and 1.7-fold in the persistent infection at 24 h p.i.), *slc1A5* (up-regulated 1.8-fold in the acute and 1.4-fold in the persistent infection at 24 h p.i.) as well as *slc19A1* (up-regulated 1.7-fold in the acute and 2.2-fold in the persistent infection at 24 h p.i.).

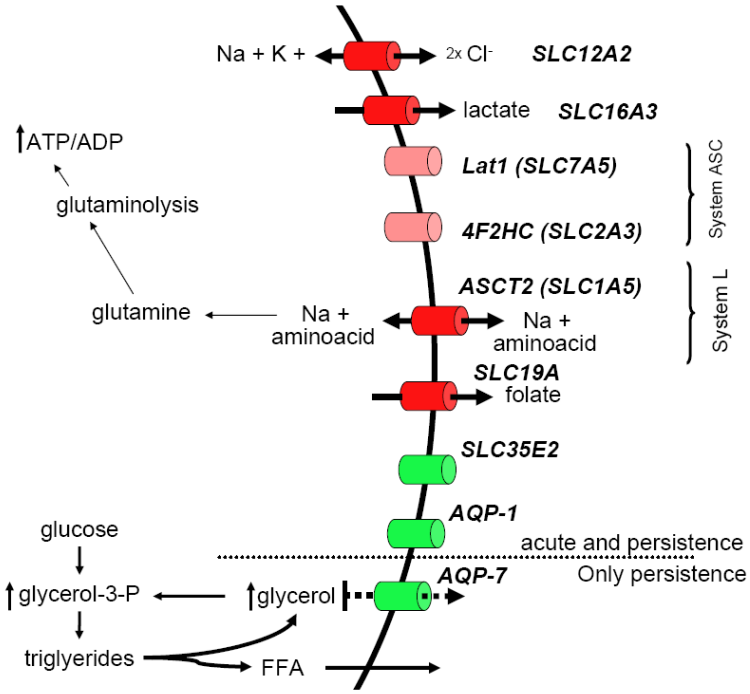


Figure 64 – ***Cpn* infection triggers the regulation of several host transporters.** This includes the up-regulation of a lactate transporter, two aminoacid transporters and one folate transporter. *AQP-7* involved in glycerol transport was down-regulated only in the persistent infection. Red color indicates up-regulation, green color indicates down regulation.

3.6.9 Acute and persistent *Cpn* infection have different effects on the host cell transcriptome

Several host genes are differentially regulated in the persistent infection but not in the acute infection and vice versa. This different impact can give an insight into the mechanisms taking part in the establishment of the persistent *Cpn* infection. As already mentioned, the kinase inhibitors *p15* and *p18* involved in cell cycle arrest were down-regulated, whereas *gadd45A* was up-regulated in the persistent infection. Differences also have been detected for the expression of *atf-3,* involved in signaling events, and several transporters including *aqp-7*, suggesting a different impact of the acute and persistent infection on cell signaling and metabolism. Moreover, *gng11* (Galpha), coding for a guanine nucleotide binding protein (G protein) was up-regulated only in the persistence. Also *rgs2***,** a regulator of G protein signaling, was induced in deferoxamine induced persistence. Also a part of the fourth component of complement (C4) system, *c4A* and *c4B* were both down-regulated in persistence.

Receptors coupled to G proteins stimulate MAPK (Lopez-Ilasaca, M. *et al.*, 1997) and also have been connected to a variety of other pathogens, like *P. falciparum* and *H. pylori* (Harrison, T. *et al.*, 2003;Wessler, S. *et al.*, 2002). *H. pylori* activates the Galpha-->cAMP-->Rap1--->B-Raf-->MEK1/2-->ERK1/2 pathway. Moreover, high cAMP levels or blocking of the MEK1/2 activity lead to a persistent infection for *Ctr* (Kaul, R. *et al.*, 1986;Su, H. *et al.*, 2004;Kaul, R. *et al.*, 1990). The RGS proteins represent a family of proteins which attenuate G protein mediated signaling. It has been shown that RGS2 is not evident in unstimulated cells but is strongly induced by one hour of treatment with forskolin, an activator of adenylatcyclase (Pepperl, D. J. *et al.*, 1998). RGS2 mRNA in peripheral blood mononuclear cells was increased in the Bartter's -Gitelman's syndrome that has been connected to hypertension (Calo, L. A. *et al.*, 2004a). It has been speculated that RGS2 is an upstream regulator of the Rho-kinase, that also plays an important role in hypertension and atherosclerosis (Calo, L. A. *et al.*, 2004b;Kanda, T. *et al.*, 2005;Lai, A. *et al.*, 2005). Antibodies to *Cpn* were found in patients with severe hypertension suggesting that this pathogen may have a role in hypertension (Cook, P. J. *et al.*, 1998).

The major functions of C4 complement are to resist infection against bacteria (Bishof, N. A. *et al.*, 1990) and to prevent immune complex disease and clusters of interlocking antigens and antibodies, which under normal conditions are rapidly removed from the bloodstream (Lachmann, P. J., 1990b;Lachmann, P. J., 1990c;Lachmann, P. J., 1990a). However, in some circumstances immune complexes work their damage in many diseases (Traustadottir, K. H. *et al.*, 2002). In some cases, as in malaria and viral hepatitis, they reflect persistent low-grade infections (Arinola, O. G., 2005;Mibei, E. K. *et al.*, 2005). *Cpn* specific circulating immune complexes were also found in patients with chronic coronary heart disease (Linnanmaki E, Leinonen M, Mattila K 1993). It also has been shown that in persons with inherent deficiency of C4 component of complement there is a predisposition for the development of diseases stimulated by Chlamydiae (Kozlov, L. V. *et al.*, 2001).

3.6.10 Discussion

The transcriptome data indicated that alternative NF-κB pathways are triggered following *Cpn* infection as *relB* - a component of the alternative pathway for NF-κB activation - as well as NF-κB2/p100 were up-regulated. RelB is able to form heterodimers with either p50 or p52 (Weih, D. S. *et al.*, 2001) protecting cells against cell death (Josson, S. *et al.*, 2005;Ryseck, R. P. *et al.*, 1996;Guerin, S. *et al.*, 2002). This led to the suggestion that *Cpn* infection triggers the processing of NF-κB2/p100 and that p65 mediated *relB* up-regulation triggers anti-apoptotic effects in the host cell. NF-κB is a coordinating factor for infection and inflammation and therefore a tight regulation of NF-κB is crucial (Bottex-Gauthier, C. *et al.*, 2002). Activation of NF-κB may be a mechanism by which cells react to stress (Wu, H. *et al.*, 1994) or trigger proliferation events (Gencay, M. M. *et al.*, 2003). Moreover, it has been described that NF-κB2/p100 and I-κBε stabilize NF-κB responses during longer stimulations (Hoffmann, A. *et al.*, 2002). RelB associated I-κBε is also involved in the induction of *ICAM-1* (Spiecker, M. *et al.*, 2000) and up-regulation *ICAM-1* has been observed in *Cpn* infected cells (Krull, M. *et al.*, 2004). The transcriptome data comfirmed also the up-regulation of *c-IAP2* and it is known, that C-IAP2 up-regulation is sufficient to suppress apoptosis (Wang, C. Y. *et al.*, 1998). Thus, it can be concluded, that NF-κB activates a group of gene products that function cooperatively at the earliest checkpoint to suppress TNF-α-mediated apoptosis. Also genes coding for MAPK3, TNF-α and the Death receptor 6 were up-regulated at 24 h post infection. It has been shown, that MAPK3 is required for TNF-induced cytokine expression (Wysk, M. *et al.*, 1999). Moreover, expression of *DR6* in mammalian cells induces apoptosis and activation of both *NF-κB* and *JNK* (Pan, G. *et al.*, 1998) and has also been observed in *Cpn* infections (Taniguchi, Y. *et al.*, 2003). In the acute infection the up-regulation of *id-1* was observed. Id-I stimulates cell proliferation (Zhang, X. *et al.*, 2004) and the growth-promoting effect of Id-1 has been demonstrated in a number of human cancers (Wong, Y. C. *et al.*, 2004). In addition, with increased *id-1* expression also increased NF-κB activity and nuclear translocation of the p65 and p50 proteins has been observed, which was accompanied by up-regulation of their downstream effectors Bcl-xL and ICAM-1 (Ling, M. T. *et al.*, 2003;Ling, M. T. *et al.*, 2004). Apoptosis, proliferation and cell signaling events are intimately coupled. The balance between cell proliferation and cell cycle arrest and cell death on the other side is very delicate (Vermeulen, K. et al., 2003a;Vermeulen, K. et al., 2003b) and decisions of the cell fate base on complex signaling networks. For *Cpn* the up-regulation of *relB* is a new finding and the involvement of the NF-κB2/p100 pathways might represent key roles to turn the balance towards anti-apoptosis and cell proliferation.

Several genes involved in host cell cycle were triggered following *Cpn* infection, including the up-regulation of *cyclin D1* and the down-regulation of *cyclin G2*. *Cyclin D1* is a NF-κB/p65 target gene (Yang, G. F. *et al.*, 2004) and NF-κB/p65 has been described to be activated following *Cpn* infection (Dechend, R. *et al.*, 1999b). Moreover, reduced *cyclin G2* expression has just recently been linked with PI3K activation (Martinez-Gac, L. *et al.*, 2004b). C*yclin G2* expression has been shown to be up-regulated as cells undergo cell cycle arrest or apoptosis (Horne, M. C. *et al.*, 1997), whereas in the G1 phase expression is decreased (Martinez-Gac, L. *et al.*, 2004a). Taken together, this suggests an PI3K influence on the regulation of these cyclins leading to a cell cycle progession mediated by *cyclin D1* up-regulation and a negative effect on cell cycle arrest by *cyclin G2* down-regulation. Indeed, this shows that Chlamydiae infected cells involve multiple signaling pathways including the NF-κB and the PI3K/AKT pathway. Overexpression of *cyclin D1* increases both phosphorylation of the retinoblastoma gene product (RB) and passage through the G1-S phase transition, resulting in increased proliferation (Grillo, M. *et al.*, 2005) and overriding cell cycle arrest (Hiyama, H. *et al.*, 1999). Further, several p53 downstream targets were affected following *Cpn* infection. *Gadd45a* is connected with the control of cell cycle checkpoints (Tong, T. *et al.*, 2005). It interacts with the Cdc2 protein kinase (Smith, M. L. et al., 1994) promoting G2/M arrest (Maeda, T. *et al.*, 2002) and has also been connected with certain types of cancer (Wang, W. *et al.*, 2005). Moreover, also another part of the activating protein transcription factor family as well as TNF-α was up-regulated following *Cpn* infection. This is consistent with a previous publication (Brenner, D. A. *et al.*, 1989) showing that TNF-α stimulates prolonged activation of the oncogene *v-JUN* expression. Thus activation of *v-Jun* gene expression may be one mechanism for mediating some of the biological effects of TNF-α up-regulation in *Cpn* infections. Beside V-*Jun* also *v-Fos* was up-regulated in the acute and persistent infection, whereas *atf-3* mRNA was only up-regulated in the acute but not the persistent infection. This is an interesting finding, as it has been described that ATF-3 antagonizes anti-apoptotic events and the inhibition of *ATF-3* up-regulation in the persistence might contribute to inforced anti-apoptosis. This is consistent with the transcriptome data showing that several cell cycle mediators were regulated in persistence but not in the acute infection. Therefore, it can be concluded that in the persistent infection additional mechanisms exist leading to an increased cell proliferation and anti-apoptotic effect.

Furthermore, several genes coding for host cell transporters were triggered following *Cpn* infection. This included a folate transporter, lactate and amino acid (AA) transporters (*asct-2* and *lat-1*) that were up-regulated and *aquaporin-1*, that was down-regulated. Moreover *aquaporin-7* (*AQP-7*) was down-regulated only in the persistent infection. The decreased expression level during adenoviral infection suggests a role for AQP1 in the abnormal fluid

fluxes detected during inflammation (Towne, J. E. *et al*., 2000). Interestingly, it has been shown that *AQP-7* knock-down leads to a decreased efflux of glycerol from the host cell and therefore increases the level of glycerol-3-phosphate and triglycerides in the host cell pool (Fruhbeck, G., 2005). Lipid levels increase during chronic *Cpn* infection (Adiloglu AK, Can R, 2005) and repeated infections with *Cpn* increased aortic sinus lipid accumulation in mice (Tormakangas L, Erkkila L 2005). As it is known that Chlamydiae use host metabolites for their metabolism (Kubo, A. *et al*., 2001b), it can be speculated that this might contribute to an increased supply of host metabolites for the pathogen in the persistence and therefore might be an important part of long-term survival of *Cpn* in the persistence. ASCT2 is a transporter, that involves the cotransport of Na^+ ions and neutral amino acids leading to an accumulation of essential amino acids (Zerangue, N. *et al*., 1996). Interestingly ASCT2 and LAT1 are up-regulated in proliferating cells (Fuchs, B. C. *et al*., 2005) and might be important for the amino acid influx observed in proliferating cells (Dransfeld, O. *et al*., 2005). Therefore this might be a result of the cell proliferation event triggered by the *Cpn* infection. In addition it might feed the pathogens need for host cell intermediates. SLC19A1 is a folate transporter. All organisms need folates for one-carbon donations and other vital functions and most bacteria synthesize them *de novo*. Folate metabolism in Chlamydiaceae has been discussed (McClarty, G., 1994;Moulder, J. W., 1991;Iliffe-Lee, E. R. *et al*., 1999) and *Chlamydia* spp. are generally susceptible to growth inhibition by sulfonamides whereas *Chlamydophila* spp. are not. The latter therefore must have mechanisms to acquire folate from the host cell, with the up-regulation of host cell transporters being one possible mechanism.

Taken together, it can be concluded that the *Cpn* infection triggers alternative NF-κB pathways, genes involved in the cell cycle and cell metabolism transport . The discovered data fit into the known chlamydial literature as anti-apoptosis, cell proliferation and the need of the pathogen for host cell intermediates is widely described. Therefore, these data showed new mechanisms involved in these processes and opens up the possibility to step closer to the pathogenic mechanisms triggered in the host cell following *Cpn* infection.

3.7 Outlook

Taken together, this study of the *Chlamydophila pneumoniae* and host cell transcriptomes led to a deeper understanding of the acute developmental cycle and the iron depletion-mediated persistent infection. The definition of cluster classes based on gene expression profiles clarified when a certain set of genes is important throughout the cycle. Consequently this characterization might be considered in future. It is also of great importance, as a lot of *Cpn* genes are still hypothetical. In combination with genome information the gene expression profiles can be used to clarify genome wide interacting networks between genes. Moreover, the microarray expression data will help to elucidate operon and promoter structures. This study was able to show that the transcriptome paves the way for different needs of the pathogen throughout the cycle. This includes genes coding for energy metabolism, as well as genes coding for membrane proteins or secretion processes. Of interest is the finding that the mRNA profile detected in elementary bodies did not show a significant overlap with a previous study characterizing its protein content. Therefore initial translation might be directed from stable transcripts present in the infectious EB. This opens up new possibilities to investigate the mechanisms connected to the onset of the chlamydial protein expression and initial time points of the infection. *In vitro* persistence models are important for the understanding how persistent Chlamydiae might act *in vivo*. DAM mediated iron depletion mimics the bodies potential to fight bacterial infections using iron withdrawal. It was demonstrated that genes showing significant differential expression in persistence were connected to either gene clusters at the beginning or the end of the acute infection. This indicated that persistence is an arrest of the gene expression in the acute mid cycle. Therefore, rather than being a new transcriptional profile, the persistence establishes because of an arrest in the 'transcriptional clockwork'. It will be of interest whether the transcriptional profile *in vivo* resembles that of the iron depletion-mediated *in vitro* model. It was demonstrated that the gene arrangement (e.g. tandemly, divergently and convergently gene pairs) influences the expression of neighboring genes. 'Transcriptional Interference' led to the down-regulation of an alternative sigma factor. Therefore the genome arrangement is of great importance for *Cpn* for establishment of the persistent infection. Moreover, it was shown that *Cpn* infection triggers host genes involved in alternative NF-κB pathways, the host cell cycle and host metabolism transport. This adds new perspectives to observations of how this pathogen might interact with its host cell and led to a deeper understanding of effects important for *Cpn* survival. Therefore this study not only gave an insight into the *Cpn* and host transcriptome in the acute and persistent infection, but will also be helpful for further investigations in this field.

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Abbreviations

AB	abberant body
AK	Aberantes Körperchen
ATP	adenosin 5´-triphosphat
bp	basepair
℃	celsius
CAD	coronary artery disease
cDNA	copyDNA
CHD	coronary heart disease
Chex	cycloheximide
Cpn	*Chlamydophila pneumoniae*
Ctr	*Chlamydia trachomatis*
CRP	C-reactive protein
d	day
DAM	deferoxamine mesylat
DNA	deoxyribonucleic acid
EB	elementary body
E. coli.	*Escherichia coli*
EK	Elementarkörperchen
EtOH	ethanol
FBS	fetal bovine serum
FITC	fluoresceinisothiocyanat
g	gram
x *g*	g force
h	hour
HDL	high-density lipoprotein
HSF	heat shock factor
Hsp	heat shock protein
H_2O	water
IL-1β	Interleukin 1β
IFNγ	interferone gamma
IFU	inclusion forming unit
IC	immuno complex
kDa	kilodalton
l	liter
LDL	low-density lipoprotein

LPS	lipopolysaccharide
m	milli
M	Molar
min	minute
MOI	multiplicity of infection
MOMP	major outer membrane protein
MS	multiple sclerosis
nm	nanometer
NTP	adenosintriphosphate
Omp	outer membrane protein
p.i.	post infection
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PCR	polymerase chain reaction
Pmp	polymorphic outer membrane protein
pH	potentia hydrogenii
RA	rheumatoid arthritis
RB	reticulate body
ReA	reactive arthritis
RNA	ribonucleic acid
rRNA	ribosomal RNA
RK	Retikularkörperchen
TTSS	Type Three Secretion System
TIISS	Type Two Secretion System
qRT-PCR	quantitative reverse transcriptase PCR
U	Unit
µ	micro

Gene names can be accessed at
http://www.stdgen.lanl.gov/stdgen/bacteria/cpneu/index.html
http://www.stdgen.lanl.gov/stdgen/bacteria/chlamy/index.html
or
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Curriculum Vitae

Personal Data

	André Germar Paul Mäurer
born	02. Dec. 1972
in	Hürth, Germany

Education

PhD student	University Würzburg, Germany. Thesis: "Microarray studies of *C. pneumoniae* and host cells during acute and iron depletion persistence." Work was performed at the Max Planck Institute for Infection Biology, Berlin, Germany as member of the Pathogenomic Network.	July 2001- current
Diploma in Biology	at the University of Würzburg, Germany Topic: Detection and discrimination of *L. pneumophila* strains via DGGE.	June 2000 - May 2001
Graduate student	at the University of Würzburg, Germany Final exams in microbiology, cell biology, and biochemistry.	until May 2000
Visiting student	at the University of Albany State /NY and School of Public Heath, Albany DAAD stipend Topic: Gene regulation in *B. subtilis* and RFLP with M. tuberculosis.	Oct. 1998 - June 1999
Graduate student	at the University of Würzburg, Germany	
Intermediate exams	at the University of Bayreuth, Germany Genetics, general biology, microbiology, chemistry, physics.	March 1996
Undergraduate student	at the University of Bayreuth, Germany	Nov. 1994 March 1996
Civil Service (alternative to military service)	at a nature preserving organization "BUND Naturschutz" in Bayreuth, Germany	Nov. 1993 - Nov 1994
Final school exams	"Gymnasium Selb"	July 1993

List of publications*:*

"Comparison of *Chlamydophila pneumoniae* genome expression profiles between acute and iron depletion-mediated persistent infection"

André P. Mäurer*, Adrian Mehlitz, Hans J. Mollenkopf and Thomas F. Meyer*

(submitted)

in preparation:

"MAPK ERK1/2 dependent regulation of TNFRI during an acute infection with *Chlamydia trachomatis*".

(manuscript in preparation)

Nicole Paland, ***André Mäurer****, Agnes Szczepek, Thomas Rudel*

"The influence of gene orientation on gene expressions levels and persistence in *C. pneumoniae* "

(manuscript in preparation)

André P. Mäurer*, Thomas F. Meyer*

"The host cell transcriptome in the acute and persistent *C. pneumoniae* infection"
(manuscript in preparation)
André P. Mäurer*, Thomas F. Meyer*

Talks and posters:

"Microarray development for the transcriptome analysis during acute and persistent infection with *Chlamydophila pneumoniae"*
Dechema, Bielefeld, Germany, Oktober 2002, http://dechema.de/bg2002

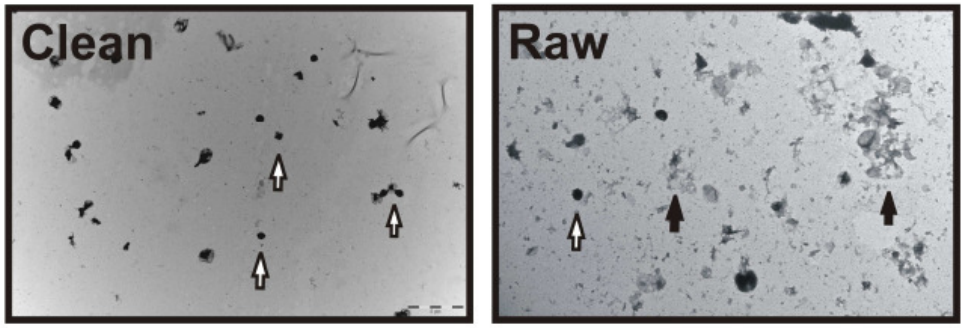
"Comparative analysis of *C. pneumoniae* transcriptome during acute and iron depletion persistent infection of human epithelial cells"
Budapest, Hungary, Sept. 2004, ISBN 963 482 666 0

"Comparative analysis of *C. pneumoniae* transcriptome during acute and iron depletion persistent infection of human epithelial cells"
IUMS, San Fransisco, USA, July 2005, 9-B-900, ISBN: 1-555-81-353-4

"Comparative analysis of *C. pneumoniae* transcriptome during acute and iron depletion persistent infection of human epithelial cells"
Dechema, Göttingen, Germany, Sept. 2005, www.dechema.de

Figure index

Table index



Supplementary-Figure 1 - **Electron microscopy of the elementary body purification.** EB were stained by negative contrasting. (A) Gradient purified Chlamydia, (B) Post nuclear supernatant after two washes with SPG before gradient purification. White arrows points out EB, black arrows show cellular components before gradient purification. Bar = 2 µm.

Supplementary-Table 1 - **Significantly regulated genes of the acute developmental cycle.** The columns are name (*Cpn* for sequences specific for strain *CWL029* and *Cp* for sequences specific for strain *AR39* or *Cpj* for sequences specific for *J138*), Cluster (according to Figure 16), regulation in DAM persistence, in EB protein composition (Vandahl, B. B. *et al.*, 2001e) and time points from 6 h p.i. to 72 h p.i. (data in log2).

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0369CP0388	12	DOWN		0.88	-0.66	-1.2	-0.51	-1.01	0	1.01	1.57
CPn0607CP0140glgC	12	DOWN		-0.06	0.24	-0.42	0.08	-0.41	0	-0.12	0.41
CPn0172guaB	12			0.25	-0.37	-0.24	0.24	-0.21	0	0.31	0.77
CPn0494CP0260	12	DOWN		0.12	-0.4	-0.95	-0.63	-0.54	0	0.96	1.73
CPn0178CP0590	12			0.61	-0.4	-0.43	-0.87	-0.35	0	0.42	0.66
CPn0537CP0215	12	DOWN		0.06	0.57	-0.64	-0.77	-1.47	0	1.42	1.94
CPn0493CP0261	12			0.84	0.24	0.2	0.46	-0.5	0	0.78	1.36
CPn0254CP0507	12			0.31	-0.14	0.11	0.05	-0.12	0	0.45	0.52
CPn1025CP0827pgi	12	DOWN		-0.18	-0.26	-0.39	-0.07	-0.21	0	0.4	1.09
CPn0065CP0709	12	DOWN		0.2	-0.04	-1.07	-0.73	-1.31	0	0.51	1.67
CPn1006CP0847	12	DOWN		1.42	-0.38	-0.98	-0.97	-1.87	0	0.98	1.75
CPn0333CP0425	12			0.59	-1.02	-2.16	-2.24	-3.39	0	2.17	2.12
CPn0728CP0018	12	DOWN	Yes	-0.63	-0.89	-0.99	-0.83	-1.37	0	0.84	1.88
CPn0162CP0609	12			0.35	-0.05	-0.28	-0.43	-0.08	0	0.16	0.69
CPn0143CP0630yxjG1	12	UP	Yes	0.14	0.18	-0.15	0.02	-0.17	0	0.1	0.64
CPn0142	12	DOWN		0.46	-0.55	-0.56	-0.56	-0.18	0	0.42	0.55
CPn0559CP0191	12			1.39	0.21	0.16	0.29	-0.59	0	0.99	2.23
CPn0040CP0735dnaX1	12			0.16	-0.93	-0.43	-0.1	0.23	0	0.48	0.36
CPn0332	12			1.73	0.31	0.79	-0.33	-1.59	0	2.29	3.37
CPn0930CP0935	12	DOWN		0.15	-0.12	-0.4	-0.2	-0.45	0	0.06	1.05
CPn0330CP0427	12	DOWN		-0.16	-0.33	-1.34	-0.57	-1.18	0	0.58	1.89
CPn0586CP0162atoC	12			0.38	-0.62	-0.02	-0.22	0.04	0	0.81	0.48
CPn0340CP0417	12	DOWN		0.23	-0.68	-0.06	-0.11	-0.7	0	0.68	1.08
CPn1023CP0829	12	DOWN		0.19	-0.37	-0.77	-0.3	-1.29	0	0.74	1.51
CPn0524CP0229	12	DOWN		0.68	-0.92	-0.99	-0.52	-1.54	0	0.74	1.5
CPn0189CP0578	12	DOWN		1.04	-0.74	-1.24	-0.41	-0.65	0	0.38	0.57
CPn0667CP0080	12			0.27	-0.91	-0.68	-0.48	-1	0	0.93	1.01
CPn0726	12			0.51	0.12	-0.47	-0.52	0.71	0	0.17	1.42
CPn0129CP0642	12			0.32	0.15	-0.56	-0.41	-0.13	0	0.29	0.44
CPn0190CP0577	12	UP		0.66	-0.25	0.6	0.28	-0.57	0	0.82	1.2
CPn0720CP0026	12	DOWN	Yes	-0.26	-0.18	-0.99	-0.48	-0.43	0	0.33	1.43
CPn0209CP0557	12			0.39	-0.35	-0.88	-0.35	-0.15	0	0.88	0.4
CPn0671CP0076	12			-0.2	-0.56	-0.69	-0.42	0.2	0	0.57	0.96
CPn0717CP0029	12			-0.15	-0.61	-0.29	-0.86	-0.27	0	0.55	0.78
CPn0724CP0022	12	DOWN		0.01	-0.2	-0.58	-0.29	-0.88	0	0.23	0.75
CPn0676CP0071	12	DOWN		-0.05	-0.94	-1.43	-1.26	-2.21	0	1.27	2.36
CPn0365CP0392	12			0.12	-0.39	-0.01	-0.67	-0.5	0	0.51	1.11
CPn0232CP0532	12			0.01	0.1	-0.74	-0.49	-0.14	0	0.73	0.34
CPn0234CP0529	12	DOWN		0.51	-0.06	0.28	0.17	-0.8	0	0.52	1.6
CPn0473CP0281	12	UP		0.49	-0.27	0.83	0.71	-0.27	0	0.52	1.7
CPn0184CP0584efp1	12		Yes	-0.23	-0.01	-0.07	0.04	0.02	0	0.18	0.33
CPn0678	12			0.84	-0.58	-0.15	-0.16	-1.57	0	1.31	2.56
CPn0974CP0885sucD	11		Yes	-0.29	-0.57	-0.66	-1.17	-1.19	0	0.1	0.06
CPn0096CP0678uvrA	11			-0.08	-0.18	-0.23	-0.45	-0.03	0	0.03	0.24
CPn0852CP1017	11	DOWN		-2.43	-2.23	-2.58	-2.25	-2.72	0	0.4	1.14
CPn0362CP0395rpsD	11	DOWN		-1.02	-1.38	-1.31	-1.54	-2.06	0	0.24	1.57
CPn0973CP0886sucC	11		Yes	-0.67	-1.76	-1.8	-1.06	-1.27	0	0.23	-0.04
CPn0809CP1062	11	DOWN		-0.73	-1.99	-3	-1.85	-2.32	0	0.88	2.25
CPn0377CP0379sucB1	11			-0.67	-1.45	-1.24	-0.75	-1.03	0	0.1	0.21
CPn0791CP1081	11	DOWN		-0.07	-1.26	-1.16	-0.98	-0.9	0	0.31	0.22
CPn0794CP1077	11	DOWN		-1.27	-1	-2.77	-2.18	-1.27	0	0.25	0.36
CPn0978CP0878	11			-0.38	-0.39	-1.03	-0.35	-0.68	0	-0.14	0.09
CPn0810CP1061	11	DOWN		-2.1	-3.09	-3.55	-2.6	-3.53	0	0.89	2.28
CPn0808CP1063	11	DOWN	Yes	-0.94	-1.73	-2.61	-1.75	-2.53	0	0.53	2.15
Cpn0016_pmp41	11	DOWN		-0.97	-0.81	-3.61	-3.02	-1.13	0	-0.13	0.28
Cpn0017_pmp42	11	DOWN		-0.08	-0.78	-0.5	-0.33	-0.53	0	0.11	0.2
CPn0811CP1060lcrH1	11	DOWN		-2.65	-2.97	-4.28	-2.41	-3.72	0	1.22	2.25
CPn0466CP0286pmp15	11			-0.01	-0.92	-0.62	-0.91	-0.33	0	-0.16	0.18
CPn0538CP0214	11	DOWN		-0.68	-1.07	-1.79	-0.64	-1.79	0	0.85	2.08
CPn0853CP1016	11	DOWN		-0.66	-1.43	-1.46	-2.03	-1.58	0	0.53	0.82
CPn0587CP0161yvyD	11			-0.2	-1.26	-1.22	-1.22	-0.86	0	0.42	1.02
CPn0324CP0433lcrE	11	DOWN	Yes	-0.49	-1.27	-1.53	-1.56	-1.27	0	-0.04	0.25
CPn0727CP0019	11	DOWN		-0.12	-0.84	-1.77	-1.54	-3.07	0	0.56	1.32
CPn0622CP0125	11			-0.37	-0.91	-1.34	-1.53	-1.2	0	-0.35	-0.25
CPn0256CP0505	11			-0.18	-0.21	-0.3	-0.44	-0.6	0	-0.29	0.02
CPn0384CP0371hctB	11	DOWN		-2.39	-3.86	-4.24	-3.26	-4.11	0	0.66	1.05
CPn0127CP0645ytfF	11			0.07	-0.37	-0.98	-0.49	-0.31	0	0.37	0.53

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0188CP0579	11	DOWN		-0.22	-0.71	-1.13	-0.9	-1.31	0	0.12	0.64
CPn0725	11	DOWN		-0.42	-1.31	-1.45	-1.17	-1.76	0	0.79	1.2
CPn0445CP0308pmp7	11	DOWN	Yes	-0.35	-1.87	-1.97	-1.25	-1.24	0	0.21	0.26
CPn0204CP0563	11	DOWN		-0.3	-0.7	-1.38	-0.6	-0.3	0	0.66	0.71
CPn0677CP0070	11	DOWN	Yes	-0.81	-1.76	-2.35	-1.59	-2.71	0	1.08	2.38
CPn0693	11	DOWN		-0.06	-0.8	-1.4	-0.84	-1.56	0	-0.01	0.79
CPn0700CP0046	11	DOWN		-0.32	-1.4	-2.08	-1.63	-1.19	0	0.25	0.38
CPn0179CP0589	11			-0.38	-0.7	-1.28	-1.3	-0.36	0	0.68	0.66
CPn0742CP0003	11	DOWN	Yes	-0.08	-1.68	-2.03	-2.08	-1.96	0	0.93	2.01
CPn0419CP0335pbp3	11			0.04	-0.52	-0.74	-0.39	-0.57	0	0.86	0.04
CPn0994CP0861	11	DOWN		-0.07	-0.4	-0.99	-0.33	-1.04	0	0.06	1.02
CPn0557CP0195omcB	11	DOWN	Yes	-2	-2.98	-3.42	-2.96	-2.74	0	0.52	1.59
CPn0558CP0193	11	DOWN		-0.98	-2.91	-3	-3.4	-2.16	0	1.09	0.72
CPn0307CP0451glgP	11	DOWN	Yes	-0.1	-0.89	-1.72	-0.38	-1.36	0	0.52	0.4
CPn0383CP0372	11			-0.06	-0.28	-0.12	-0.07	-0.31	0	-0.05	-0.01
CPn0998CP0857ftsH	11		Yes	-0.41	-0.58	-0.9	-0.5	-0.33	0	-0.09	0.46
CPn0164CP0607	11			-0.11	-0.41	-0.65	-0.85	-0.63	0	-0.01	0.73
CPn1005CP0848	11	DOWN		0.14	-1.37	-2.19	-1.81	-2.39	0	0.72	1.89
CPn0584CP0164atoS	11	DOWN		-0.09	-1	-1.59	-0.91	-1.01	0	0.11	0.1
CPn0472CP0282	11	DOWN		-0.46	-0.99	-1.32	-0.7	-1.14	0	0.51	1.78
CPn0085CP0690	11			-0.13	-0.13	-0.44	-0.66	-0.23	0	-0.01	0.24
CPn1004CP0849	11	DOWN		0.23	-1.28	-2.47	-0.62	-2.85	0	0.52	1.37
CPn0447CP0306pmp9	11			-0.21	-0.83	-0.86	-0.66	-0.48	0	0.2	0.04
CPn0194CP0573gcp2	10			-1.98	-1.84	-1.66	-1.4	-0.77	0	0.58	0.71
CPn0467CP0285pmp16	10	DOWN		-0.76	-1.82	-1.63	-1.13	-0.34	0	0.27	0.18
CPn0860CP1009fliF	10	DOWN		-2.46	-2.59	-3.21	-2.37	-1.02	0	0.64	0.4
CPn0444CP0309pmp6	10	DOWN	Yes	-1.26	-1.73	-1.64	-1.52	-0.97	0	-0.18	0.32
CPn0446CP0307pmp8	10	DOWN	Yes	-1.32	-2.98	-2.25	-1.74	-0.84	0	-0.04	0.13
CPn0854CP1015porB	10			-1.49	-1.79	-2.17	-1.18	-0.84	0	-0.04	0.43
CPn0857CP1012	10	DOWN		-0.5	-0.87	-1.65	-1.76	-0.4	0	-0.17	-0.52
CPn0125CP0648	10			-1.49	-1.23	-1.16	-1.26	-0.43	0	0.12	0.06
CPn0451CP0302pmp11	10		Yes	-0.5	-1.08	-1.83	-1.13	-0.34	0	0.42	0.21
CPn0453CP0299pmp13	10	DOWN	Yes	-2.53	-2.52	-2.26	-2.12	-0.6	0	0.16	0.38
CPn0497CP0257	10			-0.84	-1.53	-0.79	-0.63	-0.17	0	0.31	0.37
CPn0786CP1086dsbD	10			-0.59	-1	-0.96	-0.69	-0.5	0	0.03	0.31
CPn0498CP0256	10			-1.98	-2.41	-1.94	-1.26	-0.27	0	0.52	0.56
CPn0062CP0713	10			-2.11	-2.05	-1.85	-1.58	-0.37	0	0.09	0.06
CPn0769CP1103topA	10	DOWN		-0.52	-0.83	-0.6	-0.12	-0.07	0	0.08	0
CPn0056CP0719mrsA	10			-0.26	-0.76	-1.13	-0.82	-0.46	0	-0.24	-0.26
CPn0750CP1122tctD	10	DOWN	Yes	-2.31	-2.18	-2.13	-1.99	-1.27	0	0.21	-0.01
CPn0731CP0015	10			0.01	-0.01	-0.07	-0.35	-0.04	0	0.22	-0.24
CPn0777CP1095groEL2	10	DOWN		-0.59	-1.11	-0.61	-0.63	-0.62	0	0.22	-0.12
CPn0195CP0572oppA1	10	DOWN		-1.87	-2.07	-1.67	-1.5	-0.81	0	0.45	0.56
CPn0669CP0078	10	DOWN		-2.58	-1.99	-2.05	-2.08	-0.8	0	0.32	0.38
CPn0703CP0043	10			-1.67	-1.44	-1.63	-1.74	-1.05	0	0.39	0.08
CPn0774CP1098	10			-1.35	-1.61	-1.2	-1.24	-0.35	0	0.32	0.09
CPn0437CP0316clpC	10		Yes	-0.38	-0.5	-0.82	-0.91	-0.1	0	-0.18	-0.08
CPn0556CP0196crpA	10			-1.97	-1.69	-3.07	-2.05	-1.31	0	-0.41	0.38
CPn0106CP0668phoH	10		Yes	-1.16	-0.92	-1.03	-1.02	-0.12	0	0.47	0.08
CPn0103CP0671cydB	10			-1.17	-0.76	-1.54	-0.87	-0.35	0	-0.13	-0.34
CPn0105CP0669	10			-1.27	-1.54	-1.71	-1.32	-0.33	0	0.57	0.67
CPn0824CP1047yscS	10			-0.59	-0.82	-0.66	-0.59	-0.55	0	-0.33	-0.27
CPn0822CP1049	10			-1.56	-2.1	-2.8	-2.03	-0.64	0	0.36	0.52
CPn0534CP0218dksA	10		Yes	-1.96	-1.69	-1.57	-1.04	-0.71	0	0.12	-0.12
CPn0540CP0212pmp20	10		Yes	-1.7	-1.6	-1.6	-1.37	-0.47	0	-0.13	-0.11
CPn0797CP1074	10		Yes	-0.77	-1.8	-2.34	-1.29	0.08	0	-0.16	-0.36
CPn0796CP1075	10			-1.48	-3.04	-3.52	-1.74	0.77	0	-0.45	0.35
CPn0793CP1079rbsU	10			-0.73	-1.76	-1.26	-0.86	-0.18	0	-0.15	-0.63
CPn0877CP0992ybcL	10			-1.31	-1.46	-0.98	-1.01	0.01	0	0.24	0.14
CPn0701CP0045karG	10	DOWN		-0.15	-1.45	-1.87	-2.01	-0.56	0	-0.28	-0.28
CPn0971CP0888yccA2	10			-0.98	-1.4	-1.59	-1.15	-0.31	0	0.49	0.45
CPn1013CP0840fumC	10		Yes	-1.24	-1.37	-1.89	-1.13	-0.64	0	0	-0.08
CPn0933CP0928	10		Yes	-2.03	-2.55	-3.13	-2.22	-1.04	0	0.32	0.38
CPn0926CP0940	10		Yes	-1.3	-1.49	-1.67	-1.17	-0.27	0	0.23	0.18
CPn0177CP0592	10			-1.19	-2.14	-1.77	-0.77	0.09	0	0.24	0.43
CPn1016CP0837	10			-1.78	-2.26	-2.8	-1.9	-0.66	0	-0.41	0.16
CPn0355CP0404	10			-1.77	-2.08	-2.14	-1.75	-0.14	0	0.05	-0.19
CPn0133CP0639	10			-0.02	-0.24	-0.47	0	0.15	0	0.12	-0.09
CPn0399CP0356	10			-0.59	-0.8	-1.03	-0.52	-0.43	0	0.12	-0.01
CPn0001CP0775	10			-1.75	-2.93	-2.39	-2.22	0.51	0	0.33	1.14
CPn1002CP0851	10			-0.34	-1.02	-0.43	-0.52	0.02	0	0.51	0.49
CPn0160CP0611	10			-0.43	-0.79	-0.7	-0.28	-0.33	0	-0.57	-0.36
CPn0161CP0610	10			-1.1	-1.19	-1.03	-0.72	-0.46	0	-0.16	0.09
CPn1032CP0820	10	DOWN	Yes	-1.87	-2.71	-2.96	-1.71	-1.24	0	0	0.02
Cpn0015_pmp32	10			-1.36	-1.17	-1.44	-1.24	-0.39	0	-0.13	0.01

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn09691yccA1	10	UP		-0.79	-1.86	-1.47	-0.59	0.05	0	0.19	0.7
CPn0325CP0432sycE	10	DOWN		-2.24	-1.8	-2.39	-2.21	-1.07	0	-0.29	-0.18
Cpn0014_pmp31	10	DOWN		-2.77	-2.8	-2.76	-2.09	-0.38	0	0.24	0.29
CPn0323CP0434lcrD	10			-0.66	-0.72	-1.13	-0.97	-0.52	0	0.08	0.18
Cpn0018_pmp51	10	DOWN		-1.4	-1.54	-2.55	-1.55	-1.15	0	0.47	0.4
CPn0956CP0904	10	DOWN		-1.73	-1.79	-1.46	-2.05	-1.01	0	-0.23	0.16
CPn0349CP0411ytgA	10			0.02	-0.39	-0.88	-0.24	0.01	0	0.14	-0.15
CPn0350CP0409	10			-0.48	-0.22	-0.65	-0.76	0.13	0	0.16	0.29
CPn1028CP0824mdhC	10		Yes	-1.33	-1.63	-1.42	-0.98	0.06	0	-0.11	-0.16
CPn0398CP0357	10			-1.95	-2.28	-2.63	-1.95	-0.5	0	0.37	0.41
CPn1033CP0819	10	DOWN		-2.22	-3	-3.51	-3.58	-1.1	0	0.14	0.39
CPn0140CP0632yqdE	10	DOWN		-2.08	-1.24	-1.55	-1.76	-0.48	0	0.32	0.31
CPn0216CP0549	10			-0.58	-1.27	-1.8	-0.98	-0.56	0	0.02	0.08
CPn0227CP0537dsbB	10	DOWN		-1.54	-1.29	-2.49	-1.61	-1.05	0	-0.27	-0.06
CPn0139CP0633yqgE	10	DOWN		-1.83	-1.4	-1.7	-1.15	-0.57	0	0.02	0.1
CPn0894CP0972amn	10	DOWN	Yes	-1.34	-1.52	-1.52	-1.12	-0.64	0	0.21	-0.27
CPn0200CP0567oppC1	10			-0.17	-0.72	-0.44	-0.27	-0.1	0	0.09	0.12
CPn0207CP0560ybhI	10			-0.13	-1.16	-0.8	-0.24	-0.28	0	-0.1	-0.12
CPn0421CP0333yabC2	10			-0.78	-0.51	-0.64	-0.66	-0.3	0	0.21	0.14
CPn1069CP0781yfgA	10			-0.55	-1.17	-0.84	-0.73	0.2	0	0.3	0.27
CPn0886CP0980	10			-2.39	-4.06	-4.94	-3.15	-1.29	0	0.84	1
CPn0433CP0320gcsH	10			-0.65	-0.39	-0.75	-0.37	-0.28	0	-0.1	-0.11
CPn0228CP0536dsbG	10	DOWN		-2.12	-2.21	-2.9	-3.11	-1.18	0	0.03	-0.17
CPn0970CP0889tyrP2	10			-1	-1.9	-1.14	-0.67	-0.08	0	-0.21	0.1
CPn0229CP0535	10			-0.6	-0.94	-1.09	-0.23	-0.85	0	-0.16	-0.22
CPn0416CP0338himD	10	DOWN		-1.9	-3.33	-3.13	-2.45	-1.14	0	0.03	0.45
CPn0138CP0634hemL	10			-0.1	-0.26	-0.43	-0.32	0.23	0	0.04	0.15
CPn1034CP0818	10			-0.15	-0.49	-1.08	-0.27	0.25	0	0.55	0.1
CPn0735CP0011	9			-2.42	-1.61	-1.18	-0.82	0.62	0	-0.24	-0.6
CPn0687CP0059	9			-0.41	-0.43	-0.25	-0.16	0.37	0	0.18	-0.14
CPn0782CP1090tolB	9			-1.16	-0.97	-0.2	-0.26	0.22	0	0.03	0.06
CPn0785CP1087exbB	9			-1.32	-1.19	-0.35	-0.03	0.12	0	-0.21	-0.27
CPn1070CP0780	9			-0.09	-0.57	-0.1	0.34	0.07	0	0.16	-0.04
CPn0553CP0199	9			-1.57	-1.42	-0.52	-0.18	0.24	0	0.17	0.02
CPn0624CP0123gapA	9		Yes	-1.14	-1.48	-0.68	-0.76	0.23	0	-0.28	0.15
CPn0789CP1083sdhA	9			-1.47	-1.46	-0.78	-0.26	0.17	0	-0.48	-0.35
CPn0695CP0051ompA	9		Yes	-1.6	-1.85	-0.99	-0.76	0.22	0	-0.22	0.96
Cpn0061_CP0714	9		Yes	-0.62	-0.64	-0.27	-0.2	0.54	0	-0.28	-0.29
CPn0206CP0561	9			-0.76	-0.77	-0.61	-0.7	0.28	0	0.07	0.2
CPn0193CP0574argR	9			-0.57	-0.53	-0.48	-0.09	0.04	0	0.22	0.22
CPn0652CP0095lpxC	9		Yes	-0.98	-0.47	-0.25	-0.36	0.11	0	0.4	-0.16
CPn0604CP0143fliY	9			-1.17	-1.5	-0.95	-0.85	0.1	0	0.16	-0.19
CPn0754CP1118rs20	9			-0.31	-0.7	0.05	0.18	0.35	0	-0.12	0.01
CPn0594CP0154pheT	9			-1.92	-0.79	-0.01	-0.8	-0.02	0	0.52	0.41
CPn0608CP0139	9		Yes	-1.1	-0.68	-0.89	-0.53	0.42	0	0.19	0.2
CPn0262CP0499surE	9			-0.87	-0.77	-0.63	-0.09	-0.04	0	0.25	0.15
CPn0231CP0533tauB	9	UP		-0.27	-0.11	-0.22	-0.33	0.6	0	-0.09	0.18
CPn0581CP0167	9			-1.32	-1.61	-0.88	-0.48	0.37	0	0.13	-0.09
CPn0577CP0171	9			-1.71	-1.44	-0.45	-0.31	0.35	0	0.08	-0.15
CPn0286CP0472mgtE	9			-0.51	-0.2	-0.2	0.01	0.18	0	0.13	0.3
CPn1001CP0852yfhC	9	UP	Yes	-0.34	-0.42	-0.1	0.09	0.31	0	0.3	0.25
CPn0132CP0640	9			-1.4	-1.14	-0.44	-0.6	0.14	0	-0.07	-0.07
CPn0876CP0993dagA2	9			-1.41	-1.68	-1.1	-1.03	0.04	0	0.78	0.7
CPn0902CP0964nlpD	9	UP		-1.32	-1.28	0.08	0.27	0.18	0	0.2	0.15
CPn0144CP0629clpB	9		Yes	-1.79	-1.71	-0.33	-0.59	-0.08	0	0.1	0.39
CPn0901CP0965murD	9			-2.39	-1.68	-0.7	-0.42	0.09	0	0.3	0.05
CPn0936CP0925rl36	9			-0.43	-0.15	-0.47	-0.38	0.38	0	0.14	0.06
CPn0110CP0664lepB	9			-0.84	-0.35	-0.25	-0.29	0.23	0	0.05	0.02
CPn0111CP0662	9			-0.65	-0.34	-0.28	-0.26	0.25	0	0.21	-0.15
CPn0495CP0259aspC	9		Yes	-0.55	-0.61	0.05	-0.2	0.17	0	0.33	0.4
CPn0048CP0726yqfF	9			-0.86	-0.84	-0.15	-0.23	0.51	0	0.02	-0.29
CPn0963CP0897pmp21	9		Yes	-2.41	-3.27	-0.99	-0.43	0.23	0	0.04	-0.4
CPn0913CP0953	9			-1.23	-1.02	-0.16	-0.15	0.53	0	-0.14	-0.21
CPn0833CP1037	9		Yes	-0.57	-0.92	-0.59	-0.4	0.27	0	0.09	-0.15
CPn0479CP0275phnP	9		Yes	-1.46	-0.96	-0.43	0.05	0.04	0	-0.12	-0.06
CPn0112CP0661rl31	9	UP		-0.68	-1.13	-0.82	-0.7	0.52	0	-0.18	-0.13
CPn0047	9	UP		-1.06	-0.78	-0.32	0	-0.02	0	0.1	-0.33
CPn0427CP0326nqr2	9			-0.42	-0.63	-0.25	-0.31	0.21	0	0.24	-0.22
CPn0865CP1004	9			-0.71	-0.42	-0.36	-0.64	0.3	0	0.17	-0.11
CPn0337CP0421smpB	9	UP		-0.33	-0.1	-0.21	-0.25	0.19	0	0.49	0.23
CPn0872CP0997ribA/ribB	9			-1.88	-1.22	-0.39	-0.27	0.21	0	0	0.04
CPn0798CP1073	9	UP		-1.48	-1.41	-0.87	-0.93	-0.06	0	-0.06	0.09
CPn0800CP1071eno	9		Yes	-0.82	-1.11	0	-0.21	0.18	0	-0.11	-0.4
CPn0441CP0312	9			-1.51	-1.39	-0.33	-0.16	0.37	0	-0.23	-0.7
CPn0873CP0996ribE	9		Yes	-0.74	-1.06	-0.55	-0.24	0.56	0	0.09	-0.14

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0959CP0901cafE	9		Yes	-0.61	-0.67	-0.4	-0.38	0.07	0	0.2	0.22
CPn0490CP0264	9			-1.86	-1.51	-0.52	-0.54	-0.01	0	0.04	-0.43
CPn0135CP0637groES	9		Yes	-0.77	-0.67	-0.21	0.46	0.69	0	0.1	0.84
CPn0422CP0331	8		Yes	-1.45	-0.94	-0.82	-0.58	-0.35	0	0.27	0.11
CPn0248CP0513ycfV	8			-0.75	-0.43	-0.41	-0.54	-0.1	0	-0.11	-0.11
CPn0434CP0319	8			-0.6	-0.44	-0.24	-0.36	-0.13	0	-0.09	0.26
CPn0136CP0636pepF	8			-0.92	-0.3	-0.64	-0.33	0.18	0	-0.1	-0.07
CPn0250CP0511rl33	8			-0.98	-0.41	-0.41	-0.18	-0.2	0	-0.24	-0.16
CPn0423CP0330	8			-1.94	-1.01	-0.89	-1.07	-0.16	0	-0.02	-0.19
CPn0067CP0707	8			-1.17	-0.64	-1.08	-0.45	-0.15	0	-0.49	-0.34
CPn0659CP0088	8		Yes	-0.71	-0.47	-0.56	-0.44	-0.19	0	-0.07	-0.31
CPn0413CP0341msbA	8			-0.88	-0.61	-0.75	-0.26	0.14	0	-0.53	-0.38
CPn0415CP0339	8	DOWN		-1.51	-1.18	-1.08	-0.51	-0.37	0	-0.51	-0.24
CPn0661mip	8			-2.22	-2.04	-1.51	-1.01	-0.23	0	-0.2	0
CPn0134CP0638groEL1	8		Yes	-1.31	-0.89	-0.71	-1.19	-0.31	0	-0.41	0.11
CPn0670rsbW	8	DOWN	Yes	-2.17	-1.43	-1.23	-1.24	-0.53	0	0.05	0.23
CPn0684CP0062parB	8		Yes	-1.7	-0.95	-0.74	-0.48	-0.11	0	-0.05	-0.17
CPn0083CP0692tal	8			-1.13	-0.75	-0.85	-0.59	-0.5	0	-0.29	-0.15
CPn0488CP0266ycfF	8		Yes	-1.55	-0.98	-0.42	-0.72	-0.58	0	-0.74	-0.3
CPn0489CP0265	8		Yes	-1.26	-0.86	-1	-0.44	-0.13	0	-0.11	-0.14
CPn0314CP0444trxB	8		Yes	-1.41	-0.7	-0.65	-0.42	-0.35	0	-0.18	-0.27
CPn0564CP0186secD/secF	8			-1.16	-0.69	-0.73	-0.22	-0.42	0	-0.55	-0.19
CPn0301CP0457	8		Yes	-2.51	-1	-0.7	-0.66	0.17	0	-0.04	-0.03
CPn0100CP0674	8			-1.6	-0.93	-0.35	-0.24	0.06	0	-0.22	0.04
CPn0322CP0435yscU	8			-1.63	-0.81	-1	-1.05	-0.35	0	-0.23	0.08
CPn0533CP0219	8			-1.61	-0.46	-0.82	-0.5	0.06	0	-0.39	-0.32
CPn0571CP0178murA	8			-1.23	-0.62	-0.44	-0.35	-0.11	0	-0.53	-0.48
CPn0535CP0217lspA	8			-1.77	-1.42	-1.02	-0.75	-0.18	0	-0.36	-0.28
CPn0153CP0618leuS	8			-0.91	-0.25	-0.45	-0.37	0.06	0	-0.52	-0.28
CPn0546CP0206rl21	8			-0.93	-0.27	-0.29	-0.25	-0.17	0	-0.03	-0.01
CPn0295CP0463	8			-1.77	-0.56	-0.78	-0.31	-0.16	0	-0.13	-0.12
CPn0525CP0228	8		Yes	-0.32	0.23	-0.43	-0.21	0.12	0	-0.32	-0.15
CPn0260CP0501secA1	8	UP		-1.48	-0.29	-0.83	-0.48	0.14	0	-0.47	-0.26
CPn0389CP0366	8			-2.19	-0.43	-0.81	-0.88	-0.13	0	-0.34	-0.31
CPn0621CP0126ruvC	8			-1.4	-1.44	-0.8	-0.63	-0.08	0	-0.19	-0.32
CPn0616CP0131dnaB	8		Yes	-1.26	-0.59	-0.2	-0.94	-0.08	0	0.07	0.06
CPn0390CP0365ruvB	8			-1.72	-0.5	-0.68	-0.74	-0.22	0	0.4	0.6
CPn0171CP0599guaA	8			-0.9	-0.67	-0.86	-0.48	-0.32	0	-0.37	0.23
CPn0387CP0368	8			-1.06	-0.27	-0.75	-0.3	-0.49	0	0	-0.38
CPn0282CP0476xasA	8			-1.6	-0.64	-0.78	-0.75	-0.62	0	-0.87	-0.88
CPn0595CP0153	8			-1.4	-0.55	-0.24	-0.46	-0.27	0	-0.03	-0.15
CPn0104CP0670	8		Yes	-1.73	-0.72	-1.25	-0.61	0.07	0	0.19	0.07
CPn0148CP0625	8			-0.71	-0.59	-0.53	-0.28	0	0	-0.22	-0.02
CPn0154CP0617gseA	8			-1.36	-0.36	-0.82	-0.68	-0.16	0	-0.73	-0.32
CPn0547CP0205ygbB	8			-1.14	-1.34	-1.16	-0.48	0.06	0	-0.31	-0.4
CPn0702CP0044yscC	8	DOWN	Yes	-2.36	-0.98	-1.43	-1.51	-0.73	0	-0.56	-0.23
CPn0828CP1043yscJ	8			-2.02	-1	-1.01	-1.17	-1.09	0	-0.03	0.16
CPn0827CP1044	8			-2.17	-0.76	-0.86	-1.07	-0.37	0	0.08	-0.2
CPn0840CP1029	8			-2.12	-1.82	-1.13	-0.94	-0.26	0	-0.12	-0.4
CPn0834CP1035	8			-1.62	-1.11	-0.96	-0.74	-0.3	0	0.06	-0.08
CPn0825CP1046yscR	8			-1.48	-0.8	-0.81	-0.56	-0.21	0	-0.26	-0.05
CPn0846CP1023clpX	8			-1.92	-0.79	-0.47	-0.47	-0.3	0	-0.54	-0.45
CPn0826CP1045yscL	8	DOWN	Yes	-1.44	-0.92	-1.1	-1.01	-0.29	0	-0.27	-0.15
CPn0044CP0729	8			-0.85	-0.59	-0.38	-0.05	-0.51	0	0.28	-0.09
CPn0849CP1020	8	DOWN		-1.52	-1.44	-1.2	-0.89	-0.11	0	-0.17	-0.56
CPn0770CP1102	8			-1.02	-0.83	-0.6	-0.32	-0.25	0	0.02	-0.32
CPn0057CP0718sodM	8		Yes	-1.52	-0.44	-0.61	-0.5	0.26	0	-0.47	-0.4
CPn0781CP1091pal	8			-1.71	-1.29	-0.83	-0.59	-0.41	0	-0.01	0.12
CPn0778CP1094ahpC	8	UP	Yes	-1.41	-0.67	-0.36	-0.83	0.28	0	-0.62	-0.57
CPn0806CP1065thrS	8	UP	Yes	-1.29	-1.26	-0.47	-0.32	-0.45	0	0.1	-0.3
CPn0803CP1068	8		Yes	-2.65	-1.34	-0.74	-0.5	0.03	0	-0.32	0
CPn0704fliN	8			-1.8	-0.79	-0.85	-0.89	-0.73	0	-0.13	-0.2
CPn0850CP1019mreB	8			-1.59	-0.91	-0.57	-0.34	-0.21	0	-0.53	-0.39
CPn0761CP1111	8			-2.49	-1.16	-0.65	-0.56	0.03	0	-0.64	-0.38
CPn0012CP0762	8			-2.34	-1.76	-0.86	-0.45	0.12	0	-0.77	-0.52
CPn0965CP0895lpxB	8			-0.97	-1.03	-0.69	-0.52	0.04	0	-0.09	-0.43
CPn1029CP0823	8			-2.28	-1.95	-1.59	-0.95	0.17	0	-0.24	-0.15
CPn1000CP0854rs15	8			-0.53	-0.19	-0.49	-0.59	-0.24	0	-0.43	-0.13
CPn0921CP0945	8			-1.89	-0.41	-0.53	-0.94	-0.21	0	-0.72	-0.76
CPn1047CP0805dapB	8			-1.13	-0.98	-0.77	-0.52	-0.34	0	0.26	-0.2
CPn0939CP0922	8		Yes	-2.64	-1.88	-1.09	-1.11	-0.1	0	-0.41	0.22
CPn0909CP0957rsbV2	8	UP		-2.3	-1.1	-1.07	-0.73	0.36	0	-0.11	-0.23
CPn0863CP1006pgmA	8		Yes	-2.53	-1.48	-1.3	-1.02	-0.02	0	-0.11	-0.12
CPn0874CP0995	8			-0.76	-0.53	-0.49	-0.22	-0.21	0	0.24	0.02
CPn0870CP0999serS	8	DOWN	Yes	-1.9	-0.89	-0.83	-0.61	-0.36	0	-0.08	0.02

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0896CP0970	8			-1.35	-0.78	-0.47	-0.61	-0.31	0	0.24	0.2
CPn0884CP0982	8	DOWN		-1.69	-0.83	-0.79	-0.38	-0.04	0	0	-0.24
CPn0905CP0961murC/ddlA	8		Yes	-1.61	-1.1	-0.7	-0.26	0.23	0	-0.37	-0.43
CPn0899CP0967murF	8			-1.33	-0.56	-0.3	-0.46	-0.51	0	-0.01	-0.53
CPn0059CP0716dut	8			-0.76	-0.59	-0.65	-0.32	-0.25	0	-0.28	-0.2
CPn0847CP1022clpP2	8		Yes	-1.29	-0.5	-0.58	-0.21	0.01	0	-0.11	-0.31
CPn0708CP0038	8	DOWN		-1.88	-0.99	-0.87	-1.12	0.05	0	-0.24	-0.2
CPn0709	8			-2.74	-1.72	-1.77	-1.69	-0.7	0	0.02	0.37
CPn0710CP0036	8		Yes	-2.85	-1.97	-1.62	-1.71	-1.12	0	-0.65	0.12
CPn0711CP0035	8	DOWN	Yes	-3.39	-1.28	-1.39	-1.47	-0.87	0	-0.14	0.16
CPn0705CP0041	8	DOWN	Yes	-2.35	-1.1	-1.04	-1.12	-0.86	0	-0.73	-0.14
CPn0712CP0034	8	DOWN		-2.17	-0.34	-0.51	-1.15	-0.59	0	-0.5	-0.1
CPn0707CP0039yscN	8	DOWN	Yes	-2.28	-0.76	-0.56	-1.02	-0.64	0	-0.33	-0.17
CPn0713CP0033	8		Yes	-2.08	-0.92	-0.94	-1.34	-0.18	0	0.45	0.15
CPn0004CP0771gatB	7	UP	Yes	-1.34	-0.66	-0.05	0.1	0.4	0	-0.33	-0.24
CPn0263CP0498yqfU	7	UP		-1.05	-0.25	-0.48	-0.11	0.38	0	-0.19	-0.25
CPn0912CP0954	7		Yes	-1.96	-1.1	-0.28	0.11	0.45	0	-0.11	-0.38
CPn0600CP0148	7			-1.15	-0.37	-0.45	-0.3	0.2	0	-0.21	-0.12
CPn0402CP0353mutY	7			-0.69	-0.03	-0.22	-0.36	-0.01	0	-0.12	0.03
CPn0101CP0673ybbP	7			-0.74	0	0.19	-0.23	-0.01	0	0.15	0.38
CPn0668	7			-1.64	-0.25	0	-0.45	0.48	0	0.2	-0.03
CPn0401CP0354	7			-0.72	-0.04	0.22	-0.07	0.04	0	-0.42	0.12
CPn0762CP1110	7		Yes	-2.25	-0.23	-0.48	-0.26	0.18	0	-0.62	-0.28
CPn0275CP0484gyrB1	7			-1.4	0.19	-0.13	-0.31	-0.32	0	-0.5	-0.29
CPn0276CP0483	7			-1.48	0	-0.34	-0.24	0.1	0	0.01	0.23
CPn0304CP0454pdhA	7		Yes	-0.96	-0.21	-0.06	-0.05	0.37	0	0.05	-0.37
CPn0985CP0871nrdB	7	DOWN		-1.8	-0.45	-0.44	-0.84	0.13	0	0.09	0.14
CPn0309CP0449dnaA1	7			-0.4	0.03	-0.12	-0.19	-0.04	0	0.01	-0.34
CPn0338CP0419dnaN	7		Yes	-1.53	-0.07	-0.01	-0.22	0.28	0	0	-0.09
CPn0300CP0458yaeT	7		Yes	-2.11	-0.59	-0.56	-0.15	0.14	0	-0.13	-0.43
CPn0940CP0921uvrC	7			-0.75	-0.19	-0.14	-0.49	0.73	0	-0.05	0.1
CPn0297CP0461fabD	7		Yes	-1.5	-0.63	-0.29	-0.11	0.47	0	-0.39	-0.31
CPn0521CP0232glyA	7			-1.86	-0.23	-0.08	-0.04	0.45	0	-0.09	0
CPn0409CP0345	7			-1.03	-0.39	-0.08	-0.18	0.06	0	-0.33	-0.21
CPn0094CP0680valS	7			-0.78	0.02	-0.14	0.03	-0.21	0	-0.02	-0.02
CPn0520CP0233clpP1	7		Yes	-2.04	-0.41	-0.23	-0.39	-0.04	0	-0.47	-0.14
CPn0185CP0583rpe	7			-0.95	0.08	-0.21	-0.09	-0.36	0	0.26	0.26
CPn0832CP1038lipA	7			-1.42	-0.48	-0.46	-0.42	0.31	0	-0.26	-0.41
CPn0889CP0977hemN2	7			-0.97	-0.15	0.12	0.08	0.45	0	-0.09	-0.22
CPn1049CP0803lysC	7			-1.36	-0.4	-0.09	-0.25	0.06	0	-0.13	-0.35
CPn0424CP0329dnaA2	7			-1.95	-0.37	-0.32	-0.47	-0.23	0	-0.29	-0.25
CPn0448CP0305yxjG2	7			-0.47	-0.27	0.25	0.03	-0.09	0	-0.21	0.02
CPn0126CP0646	7			-0.59	-0.23	0.11	-0.07	0.36	0	-0.09	0.07
CPn0425CP0328	7			-1.93	-0.56	-0.42	-0.37	0.11	0	-0.29	-0.09
CPn0984CP0872nrdA	7	DOWN		-2.7	-0.19	-0.33	-0.66	-0.48	0	0.13	0.44
CPn0721kdsA	7			-0.87	0.04	0.02	-0.26	0.23	0	-0.04	0.09
CPn0906CP0960	7			-2.14	-0.18	-0.39	-0.45	0.24	0	-0.65	-0.23
CPn0183CP0585accB	7		Yes	-1.17	-0.15	-0.32	-0.33	0.07	0	-0.25	0.07
CPn1031CP0821arcD	7	DOWN		-3.43	-0.18	0.29	1.9	-2.31	0	-0.58	0.18
CPn0716CP0030	6	DOWN		-0.67	0.54	0.32	0.17	0.32	0	-0.51	-0.48
CPn0549CP0203rs10	6			-0.29	0.41	0.07	-0.07	0.49	0	-0.59	-0.64
CPn0551_rs7	6			-0.2	0.24	0.08	-0.17	0.31	0	-0.76	-0.65
CPn0914CP0952	6			-0.64	0.22	0.01	0.33	0.08	0	-0.12	-0.53
CPn0052CP0723hemC	6			-0.21	-0.07	-0.12	0.15	0.26	0	-0.28	-0.5
CPn0411CP0343	6			-0.28	0.41	0.61	0.76	-0.27	0	-0.8	-1
CPn0968CP0892	6			-0.66	0.26	0.01	0.23	0.13	0	-0.68	-0.65
CPn0650CP0097lpxA	6			-0.54	0.35	0.2	0.27	-0.25	0	-0.8	-0.76
CPn0658	6		Yes	-0.46	0.22	0.12	-0.21	0.51	0	-0.28	-0.65
CPn0880CP0989ftsK	6			-0.3	-0.12	0.16	0.09	0.08	0	-0.12	-0.38
CPn0487CP0267	6	DOWN		-0.54	0.22	0.23	0.05	-0.06	0	-0.36	-0.43
CPn0518CP0235	6		Yes	-0.73	1.02	0.21	0.1	0.32	0	-0.35	-0.7
CPn0077CP0698rl11	6			-0.04	0.34	-0.09	-0.15	0.23	0	-0.59	-0.46
CPn0900CP0966mraY	6			-0.08	-0.01	0.25	0.1	0.03	0	-0.17	-0.05
CPn0917CP0949	6		Yes	-0.84	0.46	0.42	0.17	0.56	0	-0.63	-0.57
CPn0891CP0975mfd	6			-0.21	0.29	0.45	0.11	-0.01	0	0.04	-0.63
CPn0532CP0220ribC	6			-0.82	0.28	-0.2	0.1	0.01	0	-0.49	-0.7
CPn0945CP0914	6			0.08	0.35	-0.17	0.03	-0.05	0	-0.86	-0.35
CPn0903CP0963ftsW	6			-0.1	-0.09	-0.08	0.42	-0.3	0	-0.15	-0.62
CPn0952CP0907rs18	6			-0.26	0.16	0.01	0.05	0.41	0	-1.07	-0.73
CPn0802CP1069trpS	6			-0.23	0.12	0.52	0.17	0.08	0	-0.21	-0.67
CPn0639CP0108rl29	6			-0.24	0.56	0.19	0.11	0.45	0	-0.76	-0.32
CPn0563CP0187recJ	6			0	-0.11	0.01	0.25	0.25	0	0.04	-0.27
CPn0612CP0135polA	6			-1.03	0.11	0.3	0.21	0.44	0	-0.87	-0.91
CPn0247CP0515rl13	6			-0.31	0.54	0	-0.13	-0.15	0	-1.06	-0.67
CPn0738CP0007	6			-0.04	-0.04	-0.2	0.02	0.17	0	-0.31	-0.46

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0613CP0134sohB	6			-0.48	-0.03	0.4	0.23	0.43	0	-0.2	-0.54
CPn0740tyrB	6			-0.37	0.63	0.19	0.22	0.06	0	-0.84	-0.57
CPn1022CP0831	6			-0.78	0.44	0.98	0.57	0.63	0	-0.21	-0.5
CPn0736CP0009ygeD	6			-0.03	0.03	0.17	0.17	0.22	0	-0.45	-0.78
CPn1021CP0832lcrH2	6			-0.55	0.47	1.19	0.52	0.13	0	-0.87	-0.8
CPn1017CP0836lytB	6			-0.8	-0.17	-0.02	0.09	0.34	0	-0.69	-0.74
CPn0603CP0144hemZ	6			-0.19	-0.18	0.42	0.26	-0.28	0	-0.77	-0.86
CPn0619CP0128ndk	6		Yes	-0.83	0.49	0.48	0.52	0.14	0	-0.62	-0.72
CPn0733CP0013rs4	6			-0.36	0.83	-0.21	-0.2	0.37	0	-0.74	-0.66
CPn0756CP1116rpoD	6			-0.41	0.43	0.54	0.37	-0.08	0	-0.27	-0.86
CPn0638CP0109rs17	6			-0.17	0.3	0.08	0.27	0.13	0	-0.32	-0.46
CPn1068CP0782rnhB2	6			-0.15	0.28	0.16	0.06	0.07	0	-0.66	-0.43
CPn0640CP0107rl16	6			-0.31	0.04	0.23	0.17	0.36	0	-0.54	-0.37
CPn0729CP0017	6			-1.15	0.59	0.81	0.68	0.73	0	-0.48	-0.74
CPn0186CP0581	6			-0.31	-0.35	0.27	0.21	0.4	0	-0.73	0.08
CPn1060CP0790tktB2	6			-0.18	0.07	0.4	0.07	0.36	0	-0.52	-0.37
CPn0626CP0121rpoA	6		Yes	-0.18	0.56	0.34	0.04	-0.14	0	-0.85	-0.59
CPn0631CP0116rs5	6	DOWN		0.12	-0.02	0.29	0.21	0.12	0	-0.55	-0.56
CPn0692CP0054	6			-0.15	0.15	0.44	0.25	0.09	0	-0.21	-0.47
CPn1042CP0810bioD	6			-1.02	0.65	0.83	0.52	0.26	0	-0.35	-0.5
CPn0753CP1119	6	UP		-0.06	-0.04	0.37	0.03	0.38	0	-0.62	-0.37
CPn0605CP0142yhhF	6			-0.62	0.41	0.72	0.24	0.11	0	-0.56	-0.82
CPn0990CP0865infC	6	UP		-0.33	0.33	0.14	0.05	0.26	0	-0.21	-0.4
CPn0569CP0180plsC	6			-0.56	0.55	-0.01	0.18	0.42	0	-0.51	-0.61
CPn0298CP0460fabH	6		Yes	-0.15	-0.06	0.04	0	0.1	0	-0.14	-0.22
CPn0991CP0864rl35	6			-0.5	0.65	0.02	0.07	0.29	0	-0.67	-0.68
CPn0574CP0175	6			-0.63	0.12	0.69	0.24	0.4	0	-0.47	-0.51
CPn0758CP1114folP	6		Yes	-1	0.21	0.29	0.45	0.56	0	-0.66	-0.85
CPn0316CP0441nusA	6		Yes	-0.02	0.23	-0.1	0.11	-0.18	0	-0.67	-0.49
CPn0327CP0430rl28	5			-0.02	0.41	0.18	0.12	0.45	0	-0.06	-0.43
CPn0317CP0440infB	5	DOWN		-0.09	0.86	0.18	0.32	0.46	0	-0.73	-0.57
CPn0428CP0325nqr3	5			0.27	0.02	0.14	0.28	-0.23	0	-0.27	-0.29
CPn0848CP1021tigA	5		Yes	0.15	1.01	0.87	1.12	0.45	0	-0.68	-0.59
CPn0966CP0894pcnB2	5			0.04	0.61	0.49	0.06	0.06	0	-0.55	-0.7
CPn0890CP0976hemE	5			-0.23	0.31	0.55	0.46	0.35	0	0	-0.63
CPn1044CP0808bioB	5			-0.58	0.78	0.75	0.72	0.52	0	-0.14	-0.26
CPn1043CP0809bioF2	5			-0.26	0.63	0.97	0.62	0.07	0	-0.1	-0.6
CPn0440CP0313	5			0.64	1.74	0.93	1.57	-0.13	0	-0.84	-0.88
CPn0321CP0436ychF	5		Yes	-0.39	0.79	0.04	0.29	0.15	0	-0.5	-0.45
CPn0312CP0446	5			0.31	0.49	-0.11	0.09	0.26	0	-0.33	-0.18
CPn0892CP0974alaS	5			-0.17	0.21	0.74	0.6	0.34	0	-0.23	-0.32
CPn0688CP0058	5			0.26	0.16	0.21	0.29	-0.23	0	-0.46	-0.68
CPn0643CP0104rs19	5			0.1	0.61	0.44	0.47	0.46	0	-0.64	-0.15
CPn0916CP0950fabF	5		Yes	0.19	0.11	0.03	0.43	0.33	0	-0.17	-0.4
CPn0989CP0866	5			0.54	0.46	0.46	0.38	-0.07	0	-0.7	-0.41
CPn0235CP0527kdsB	5		Yes	0.1	0.06	0	0.23	0.05	0	-0.37	0.05
CPn0283	5			0.21	0.45	0.09	-0.03	0.02	0	-0.42	-0.22
CPn0993CP0862pheS	5			0.02	1.08	0.75	0.65	-0.21	0	-0.27	-0.53
CPn0279CP0479	5	UP		-0.38	0.65	0.46	0.43	0.35	0	-0.1	-0.07
CPn1015CP0838	5	UP		-0.21	0.5	0.18	0.26	-0.2	0	-0.21	-0.47
CPn0992CP0863	5			0.12	1.01	0.4	0.17	0.54	0	-0.44	-0.67
CPn0915CP0951ybeB	5			0.18	0.07	0.18	0.25	-0.21	0	-0.52	-0.47
CPn0318CP0439rbfA	5	DOWN		-0.07	1.53	0.5	0.39	0.5	0	-0.78	-0.77
CPn0289CP0469	5			-0.42	0.77	0.57	0.42	0.41	0	-0.34	-0.29
CPn0364CP0393fer4	5			-0.24	0.45	0.51	0.54	0.15	0	-0.15	-0.24
CPn0238CP0524zwf	5			0.13	0.32	0.23	0.17	0.53	0	-0.3	-0.31
CPn0346CP0414ytgD	5			0.42	0.67	0.34	0.12	0.23	0	-0.58	-0.26
CPn0953CP0906rl9	5		Yes	0.29	0.8	0.39	0.35	0.63	0	-0.62	-0.56
CPn0251CP0510	5			-0.3	1.29	0.34	0.58	0.27	0	-0.38	-0.72
CPn0690CP0056	5		Yes	0.02	0.45	0.56	0.26	0.39	0	-0.34	-0.61
CPn0352CP0407	5			0.21	-0.12	0.27	0.37	0.18	0	-0.29	-0.39
CPn0950CP0909pth	5			0.4	1.48	0.72	0.58	-0.09	0	-0.82	-0.38
CPn0121CP0652	5		Yes	-0.22	0.99	0.37	0.15	0.51	0	-0.43	-0.35
CPn0644CP0103rl2	5			0.27	0.38	0.61	0.09	0.49	0	-0.72	-0.28
CPn0091CP0683atpI	5			0.18	0.91	0.61	0.63	0.32	0	-0.54	-0.34
CPn0081CP0694rpoB	5	DOWN		0.36	1.27	1.09	0.61	0.94	0	-0.9	-0.72
CPn0115CP0658ffh	5		Yes	0.38	1.13	0.25	0.36	0.29	0	-0.78	-0.41
CPn0550fusA	5		Yes	-0.02	1.09	0.36	0.64	0.54	0	-0.76	-0.66
CPn0079CP0696rl10	5		Yes	0.53	0.48	0.09	0.26	-0.08	0	-0.7	-0.42
CPn0575CP0174yhhY	5			-0.32	0.67	0.43	0.53	-0.3	0	-0.54	-0.29
CPn0647CP0100rl3	5			-0.07	0.5	0.39	0.22	-0.02	0	-0.37	0.04
CPn0645CP0102rl23	5			-0.07	0.25	0.23	0.21	0.52	0	-0.31	-0.17
Cpn0548_CP0204	5			0.37	0.82	0.71	0.54	0.7	0	-0.6	-0.82
CPn0611CP0136yacE	5		Yes	0.08	0.45	0.35	0.52	-0.02	0	-0.16	-0.34
CPn0759CP1113folA	5			0.14	0.62	0.83	0.63	0.38	0	-0.46	-0.77

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0504CP0249vacB	5			0.56	0.68	0.08	0.26	0.14	0	-0.54	-0.62
CPn0088CP0686atpA	5			-0.05	0.92	0.38	0.45	0.41	0	-0.47	-0.19
CPn0075CP0700secE	5			-0.03	1.06	0.08	0.52	-0.01	0	-0.26	-0.67
CPn0734yceA	5			0.05	0.45	0.36	0.38	0.53	0	-0.32	-0.49
CPn0076CP0699nusG	5		Yes	0.1	0.92	0.45	0.31	0.25	0	-0.64	-0.31
CPn0539CP0213pmp19	5	UP		-0.32	0.76	0.81	0.53	0.05	0	-0.03	-0.45
CPn0632CP0115rl18	5	DOWN		0.05	0.26	0.33	0.29	0.62	0	-0.6	-0.6
CPn0635CP0112rl5	5			-0.14	0.32	0.22	0.28	0.3	0	-0.57	-0.26
CPn0050	5			0.51	0.88	0.04	-0.46	-0.07	0	-0.67	-0.6
CPn0575aprfB	5			-0.35	0.74	0.57	0.39	-0.16	0	-0.37	-0.38
CPn0634CP0113rs8	5	DOWN		0.04	0.47	0.34	0.27	0.14	0	-0.72	-0.38
CPn0633CP0114rl6	5	DOWN		0.13	0.97	0.45	0.44	0.26	0	-0.7	-0.51
CPn0630CP0117rl15	5			0.02	0.58	0.2	0.67	0.04	0	-0.69	-0.55
CPn0625CP0122rl17	5			-0.02	0.61	0.55	0.16	0.1	0	-0.68	-0.52
CPn0646CP0101rl4	5			-0.1	0.6	0.27	0.42	-0.1	0	-0.63	-0.43
CPn0543CP0209	5			-0.01	1.32	0.69	0.36	0.29	0	-0.56	-0.77
CPn0116CP0657rs16	5			0.26	0.61	0.38	0.17	0.44	0	-0.72	-0.62
CPn0628CP0119rs13	5		Yes	0.07	0.7	0.54	0.17	0.45	0	-0.74	-0.45
CPn0092CP0682atpK	5			0.04	0.32	0.47	0.47	0.47	0	-0.4	-0.26
CPn0570CP0179	4			0.1	0.26	0.72	0.58	0.59	0	0.04	-0.61
CPn0972CP0887ftsY	4		Yes	0.07	0.24	0.79	0.14	0.6	0	-0.04	-0.54
CPn0093CP0681	4			-0.05	0.61	0.52	0.32	0.37	0	-0.13	-0.22
CPn0344CP0416yaeL	4			-0.06	0.36	0.2	-0.02	-0.18	0	0.15	-0.22
CPn0351CP0408adt1	4			0.57	1.09	0.08	-0.1	0.26	0	0.07	-0.11
CPn0542CP0210	4			0.86	0.84	0.59	0.71	0.06	0	-0.2	-0.33
CPn0544CP0208yhbZ	4			0.62	0.97	0.37	0.14	0.42	0	0	-0.23
CPn0983CP0873	4			0.14	0.25	0.35	0.09	0.25	0	0.18	-0.48
CPn0649CP0098fmt	4	DOWN		0.51	1.32	1.06	0.58	0.23	0	-0.02	-0.4
CPn0078CP0697rl1	4			0.21	0.4	0.3	0.28	0.46	0	-0.16	-0.07
CPn0055CP0720	4			0.39	0.47	0.5	0.37	0.09	0	-0.43	-0.29
CPn0560CP0190gltX	4			0.6	1.09	1.11	0.68	0.39	0	-0.08	-0.39
CPn0319CP0438truB	4			0.12	0.27	0.37	-0.03	0.54	0	0	-0.07
CPn0120CP0653gmk	4		Yes	0.29	1.06	0.4	0.32	0.59	0	-0.49	-0.15
CPn0689CP0057yfhO1	4			0.43	0.98	0.93	0.75	0.58	0	-0.14	-0.3
CPn0058CP0717accD	4			0.58	0.8	0.08	0.15	0.49	0	-0.32	-0.34
CPn0578CP0170yaeI	4			-0.09	0.17	0.67	0.5	0.62	0	0.12	-0.38
CPn1040CP0812	4		Yes	0.81	0.94	0.96	1.02	0.29	0	-0.14	-0.49
CPn0237CP0525yggF	4			0.46	0.82	0.51	0.57	0.41	0	-0.48	-0.27
CPn0236CP0526pyrG	4			0.76	0.53	0.42	0.56	0.41	0	-0.5	-0.19
CPn0629CP0118secY	4	DOWN		0.41	0.77	0.75	0.52	0.17	0	-0.25	-0.33
CPn0230CP0534	4			0.21	0.4	0.24	0.23	0.42	0	-0.16	-0.11
CPn0080CP0695rl7	4		Yes	0.89	0.84	0.16	0.16	0.21	0	-0.36	-0.58
CPn1051CP0801	4			0.22	0.34	0.39	0.6	0.09	0	-0.15	-0.51
CPn1059CP0791kgsA	4			0.36	0.86	0.53	0.31	-0.09	0	-0.11	-0.82
CPj0954Cpn0954	4			0.81	0.74	0.87	0.65	0.75	0	-0.23	-0.22
CPn0739CP0006	4			0.89	1	0.78	0.23	0.4	0	0.36	-0.34
CPn0617CP0130gidA	4			1.06	1.37	1.1	0.73	0.84	0	-0.21	-0.4
Cpn0090_CP0684	4			0.22	0.77	0.93	0.85	0.22	0	-0.5	-0.27
CPn0087CP0687	4			0.58	1.11	0.71	0.72	0.36	0	-0.18	-0.18
CPn0290CP0468	4			0.05	1.07	0.91	0.68	0.5	0	0.25	-0.04
CPn0089CP0685atpB	4		Yes	0.26	1.18	0.77	0.49	0.37	0	-0.18	-0.33
CPn0598CP0150oppB2	4			0.31	0.36	0.13	-0.06	0.59	0	-0.11	-0.48
CPn0597CP0151oppC2	4			0.36	0.52	0.64	0.64	0.26	0	-0.41	-0.32
CPn0741CP0004	4			0.43	1.36	0.62	0.71	0.28	0	-0.54	-0.59
CPn0601CP0146	4			0.17	0.43	0.86	0.45	0.66	0	-0.17	-0.24
CPn0003CP0772gatA	4		Yes	0.18	0.65	0.67	0.78	0.37	0	-0.29	-0.33
CPn0359CP0399lepA	4			0.58	0.64	0.67	0.47	0.24	0	-0.53	-0.3
CPn0961CP0899rl32	4	UP		0.56	1.35	0.75	0.49	0.7	0	-0.11	-0.46
CPn0674CP0073fmu	4			0.64	1.65	1.56	0.78	0.16	0	-0.31	-0.29
CPn0109CP0665ileS	4			0.01	0.31	0.38	0.14	0.37	0	-0.21	0
CPn0119CP0654rnhB1	4			0.63	0.87	0.49	0.43	0.45	0	-0.35	-0.33
CPn0942CP0919dnaG	4			0.13	0.07	0.23	0.32	0.36	0	0.19	-0.35
CPn0122CP0651metG	4		Yes	0.51	0.83	0.36	0.53	0.15	0	-0.41	-0.41
CPn0412CP0342	4			0.84	0.81	0.59	0.49	0.35	0	-0.42	-0.53
CPn0476CP0278	4			0.78	0.78	0.8	0.44	0.54	0	-0.15	-0.29
CPn0410CP0344dnaQ1	4			0.21	0.32	0.26	0.21	0.25	0	-0.15	-0.29
CPn0910CP0956miaA	4			0.13	0.35	0.5	0.38	0.45	0	0	-0.17
CPn0719CP0027sfhB	4			0.27	0.39	0.65	0.32	0.41	0	-0.2	-0.17
CPn0829CP1042	4	UP		0.35	0.41	0.14	0.33	-0.06	0	0.17	-0.5
CPn0118CP0655rl19	4			0.45	1.25	0.59	0.64	0.35	0	-0.47	-0.22
CPn0397CP0358	4			0.33	0.86	0.76	0.33	0.49	0	-0.17	-0.11
CPn0839CP1030psdD	4			0.4	0.93	0.55	0.47	0.23	0	-0.15	-0.39
CPn0114CP0659hemK	4			0.54	0.81	0.55	0.58	-0.03	0	-0.18	-0.08
CPn0071CP0704	4			0.09	0.52	0.2	0.38	-0.28	0	0.1	-0.13
CPn0037CP0738ptsH	4			0.16	0.39	0.48	0.19	0.2	0	-0.16	-0.04

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0032CP0744dnaJ	4		Yes	0.95	1.29	0.44	0.3	0.3	0	-0.17	-0.22
CPn0117CP0656trmD	4			0.57	1.27	0.83	0.69	0.65	0	-0.41	-0.48
CPn0836CP1033brnQ	4			0.17	0.55	0.45	0.3	0.13	0	-0.15	-0.17
CPn0835CP1034	4			0.74	1.72	0.97	0.78	0.46	0	-0.28	-0.82
CPn0527CP0225sucB2	4		Yes	0.56	0.64	0.37	0.55	0.13	0	-0.27	-0.45
CPn0655CP0092dnaQ2	4	DOWN		0.54	0.99	0.85	0.45	-0.04	0	-0.25	-0.35
CPn0801CP1070	4			0.38	0.63	0.58	0.66	0.62	0	-0.08	-0.4
CPn0023CP0753yjjK	4		Yes	0.71	1.01	0.64	0.62	0.1	0	-0.42	-0.32
CPn0927CP0939	3			1.5	1.22	1.51	1.02	-0.07	0	-0.03	-0.22
CPn1003CP0850	3			0.15	0.18	0.48	0.55	-0.1	0	0.5	-0.46
CPn0145CP0628	3			0.56	0.38	0.67	0.31	0.1	0	0.27	0.13
CPj05762CPn0576	3			0.41	-0.02	1.55	0.79	0.08	0	0.12	0.28
CPn0686CP0060	3			0.7	0.39	0.58	0.8	0.41	0	0.16	0.01
CPn0541CP0211	3			0.54	0.71	0.78	0.36	0.39	0	0.12	0.06
CPn1063CP0786tpiS	3	UP	Yes	0.76	0.7	0.68	0.46	0.38	0	0.1	0.04
CPn0589CP0159	3	DOWN		0.09	0.37	0.58	0.56	-0.21	0	0.06	0.01
CPn0456	3			1.13	0.42	0.79	0.68	0.39	0	0.39	-0.06
CPn0025CP0751atsA	3	UP		1.2	1.57	1.44	1.11	0.32	0	0.39	-0.05
CPn0264CP0497ubiD	3	UP		0.59	0.56	1.15	0.69	0.19	0	0.55	0.19
CPn0746CP1126	3		Yes	0.28	0.07	0.44	0.16	0.23	0	0.32	-0.46
CPn0150CP0623	3			1.63	0.31	1.78	1.06	0.05	0	0.18	0.21
CPn0934CP0927rnpA	3			0.53	0.31	0.73	0.16	0.06	0	0.25	0.06
CPn0358CP0400	3	UP		0.68	0.06	0.95	-0.14	0.63	0	0.16	-0.37
CPn0871CP0998ribD	3	UP		0.57	0.25	0.75	0.56	0.33	0	0.4	-0.08
CPn0641CP0106rs3	3			0.49	0.35	0.34	0.45	0.58	0	0.38	0.17
CPn0147CP0626	3			0.05	0.52	1.05	0.58	0.27	0	-0.22	0.21
CPn0627CP0120rs11	3			0.23	0.31	0.43	0.36	-0.01	0	-0.3	0.15
CPn0881CP0986	3			1.54	1.14	1.93	1.73	0.24	0	0.21	-0.5
CPn1065	3	UP		0.64	-0.12	0.63	0.9	-0.38	0	0.36	-0.05
CPn0026CP0750	3			1.86	1.65	2.01	1.2	0.74	0	0.36	0.06
CPn0356CP0402	3			0.32	0.03	0.96	0.6	0.28	0	0.24	-0.08
CPn0146CP0627	3			1.16	1.2	1.26	0.95	0.04	0	0.04	0.14
CPn0491CP0263	3			0.73	0.27	0.68	0.49	-0.04	0	0.22	0.01
CPn0068CP0706	3			1.79	3.21	3.31	1.54	-0.12	0	0.42	1.83
CPn0463CP0289	3			1.41	0.25	1.47	0.85	0.33	0	1.05	0.06
CPn0379CP0377	3			0.38	1.2	1.09	0.77	0.05	0	-0.03	0.6
CPn0418CP0336murE	3	UP		1.82	1.9	2.13	1.36	0.21	0	0.18	0.28
CPn0554CP0198	3	UP		1.12	0.54	1.04	0.63	0.55	0	0.08	0
CPn0191CP0576glnQ	3			0.17	0.7	0.93	0.79	0.42	0	0.26	0.2
CPn0315CP0442	3		Yes	0.51	0.04	0	1.52	0	0	-0.03	-0.05
CPn0530CP0222spoU1	3			0.47	0.4	0.77	0.73	-0.15	0	0.42	-0.2
CPn0396CP0359yhfO	3			1.19	1.02	1.25	0.78	0.46	0	0	0.13
CPn0211CP0554	3	UP		1.44	0.61	1.87	0.84	0.21	0	0.23	0.44
CPn0054CP0721rnc	3		Yes	0.46	0.32	0.51	0.51	0.48	0	0.12	-0.04
CPn0813CP1058pepP	3		Yes	0.1	-0.34	0.65	0.38	0.5	0	0.26	0.15
CPn0723yhbG	3	UP		0.69	0.89	1.14	0.62	0.29	0	-0.11	0.36
CPn1054CP0764	3		Yes	0.28	-0.31	0.52	0.55	0.36	0	-0.21	-0.23
CPn0986CP0869yggH	3	UP		0.22	0.37	1.02	0.67	0.1	0	0.15	-0.22
CPn1053CP0799	3			0.66	1.34	1.37	0.66	0.31	0	-0.05	0.02
CPn0979CP0877htrA	3	UP	Yes	-0.12	-0.18	0.88	0.03	0.45	0	-0.13	0.27
CPn0292CP0466incC	3			-0.03	0.24	0.61	0.67	0.33	0	-0.16	-0.01
CPn0051	3			0.47	0.14	0.74	-0.05	-0.13	0	0.03	0.01
CPn0680CP0067ygo4	3			0.9	0.61	0.92	0.4	0.18	0	0.46	-0.22
CPn0787CP1085yabD	3	UP		1.03	0.89	1.42	1.03	0.29	0	0.28	0.09
CPn0962CP0898plsX	3			0.2	0.75	0.81	0.49	0.09	0	0.17	0.27
CPn0975CP0882	3			0.37	0.32	0.51	0.39	-0.38	0	-0.14	0.3
CPn0867CP1002rodA	3	UP		0.88	0.21	1.03	0.7	0.28	0	0.41	0.55
CPn0043CP0730	3	UP		1.28	0.87	1.37	0.76	0.27	0	0.38	0.29
CPn1052CP0800	3	UP		0.19	4.02	3.33	1.8	-0.45	0	0.45	0.76
CPn0403yceC	3	UP		1.15	0.72	1.31	0.65	0.63	0	0.5	0.17
CPn0924CP0942priA	3			1.2	0.96	1.31	0.63	0.23	0	0.31	-0.15
CPn0035CP0741	2	UP		3.28	2.31	1.74	1.15	0.08	0	0.53	0.52
CPn0066CP0708	2	UP		2.49	1.92	1.74	1.24	0.06	0	0.74	0.81
CPn0187CP0580	2			2.28	1.52	1.2	0.88	0.47	0	0.67	0.65
CPn0020CP0756	2	UP		1.82	0.63	1.17	1.06	0.18	0	0.09	0.19
CPn0030CP0746gcp1	2			2.87	1.52	0.76	0.29	0.31	0	0.19	0.24
CPn0024CP0752xerC	2	UP		2.88	1.28	1.3	1.2	0.17	0	0.49	0.45
CPn0049CP0725	2			0.84	0.86	-0.22	0.16	0.08	0	-0.27	-0.07
CPn0095CP0679	2			0.64	0.3	0.6	0.24	0.02	0	0.02	0.19
CPn0098htrB	2			2.9	1.74	0.62	0.68	-0.17	0	0.64	0.6
CPn0034CP0742	2	UP		0.63	0.24	0.45	0.34	0.41	0	0.3	0.25
CPn0102CP0672cydA	2	UP		2.12	1.23	1.09	0.8	0.44	0	0.45	0.49
CPn0152CP0620	2			0.95	0.89	0.01	0.51	0.36	0	-0.1	0.06
CPn0149CP0624dnlJ	2	UP		3.14	2.44	1.23	1.34	0.41	0	0.88	0.86
Cpn0019_pmp52	2			1.53	0.89	1.13	0.93	-0.41	0	0.9	0.15

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0046CP0728	2	UP		1.57	0.98	1.11	0.75	0.19	0	0.01	0.08
CPn0163CP0608	2	UP		1.69	0.32	0.52	0.62	0.03	0	0.46	0.75
CPn0041CP0733	2			1.36	0.33	0.23	0.61	-0.24	0	0.21	0.3
CPn0113CP0660pfrA	2		Yes	1.11	1.07	1.01	0.39	0.62	0	0.16	-0.15
CPn0006	2	UP		2.22	1.25	1.31	0.67	0.31	0	1.01	0.56
CPn0182CP0586accC	2			1.2	0.93	0.44	0.73	0.21	0	-0.08	0.04
CPn0036CP0739	2			1.04	0.9	0.89	0.69	0.23	0	0.15	0.15
CPn0038CP0737ptsI	2	UP		2.45	1.15	1.76	0.51	0.13	0	0.05	0.82
CPn0008CP0766	2	UP		1.26	0.71	0.72	0.24	0.71	0	0.02	0.19
CPn0009CP0765	2	UP		1.33	0.89	1.38	0.39	0.92	0	0.6	0.58
CPn0086_CP0688	2	UP		2.11	1.68	1.18	1	0.35	0	0.17	0.3
CPn0168CP0603	2	UP		2.45	1.03	1.37	0.68	0.01	0	0.15	0.68
CPn0167CP0604	2			1.56	0.63	0.63	0.3	-0.23	0	0.2	0.51
CPn0028CP0748	2	UP		1.89	1.05	1.02	0.66	0.15	0	0.29	0.27
CPn1073CP0776	2	UP		2.45	1.25	1.13	0.74	0.17	0	0.29	0.44
CPn0196CP0571oppA2	2			1.3	1.19	0.61	0.17	-0.36	0	0.63	0.24
CPn0197CP0570oppA3	2			1.03	0.93	0.45	0.39	0.04	0	0.23	0.27
CPn0618CP0129lplA2	2	UP		2.29	1.69	1.48	1.2	0.18	0	0.79	0.29
CPn0593CP0155	2			1.57	0.6	0.75	0.35	0.14	0	0.46	0.33
CPn0596CP0152ada	2			2.35	1.16	1.41	1.03	0.14	0	0.64	0.2
CPn0614CP0133adt2	2			1.96	1.53	1.38	0.93	0.16	0	-0.24	0.16
CPn0615CP0132pgsA1	2			1.57	1.09	0.42	0.33	0.04	0	0.34	0.08
CPn0620CP0127ruvA	2	UP		1.67	0.61	1.05	0.64	0.29	0	0.48	0.33
CPn0588CP0160	2	UP		2.76	1.21	1.31	0.75	-0.14	0	0.71	1.05
CPn0648CP0099	2			2.39	1.79	1.4	0.93	0.68	0	-0.07	0.4
CPn0656CP0091	2			0.8	0.64	0.19	-0.41	-0.08	0	-0.27	0.07
CPn0673CP0074	2			2.4	1.42	1.58	0.68	0	0	0.43	0.16
CPn0679CP0068	2	UP	Yes	2.02	1.47	1.84	0.94	0.7	0	0.4	0.5
CPn0590CP0158	2			0.66	0.72	0.34	0.02	0.05	0	0.44	0.1
CPn0580CP0168truA	2	UP		1.84	1.23	1.42	0.73	0.49	0	0.06	0.29
CPn0585CP0163	2			1.64	1.46	1.22	0.46	0.18	0	-0.11	0.11
CPn0715CP0031gyrB2	2			2.39	2.08	1.15	0.93	0.21	0	0.47	0.12
CPn0529CP0223ycaH	2			0.75	0.69	0.32	0.49	-0.08	0	0.24	0.07
CPn0509CP0245	2			0.91	0.86	0.59	0.62	0.15	0	0.05	-0.06
CPn0515CP0238ubiE	2			1.56	0.56	1.09	0.97	0	0	0.14	0.06
CPn0522CP0231	2	UP		2.65	1.4	1.45	1.02	0.31	0	0.67	0.38
CPn0523CP0230	2			2.78	1.74	1.39	0.42	-0.12	0	0.24	0.37
CPn0536CP0216dagA1	2			1.16	0.45	0.81	0.53	0.33	0	0.11	-0.13
CPn0579CP0169yacM	2			2.91	1.98	1.56	0.98	0.08	0	-0.2	0.27
CPn0198CP0569oppA4	2			1.9	0.99	0.72	0.62	0.45	0	0.39	0.4
CPn0562CP0188	2			1.71	1.3	0.68	0.52	0.22	0	0.3	0.47
CPn0565CP0185	2			2.42	1.18	0.95	0.82	-0.07	0	1.02	0.74
CPn0572CP0177	2		Yes	2.65	1.43	0.79	0.91	-0.17	0	0.65	0.88
CPn0682CP0065dppD	2	UP		3.07	1.69	1.45	1.1	0.64	0	0.54	0.18
CPn0766CP1106	2			2.65	1.3	1.27	1.01	0.68	0	0.31	0.2
CPn0730CP0016mviN	2	UP		1.5	0.9	0.76	0.91	0.17	0	0.5	0.13
CPn0499CP0255	2	UP		2.47	1.69	1.19	1.22	0.31	0	0.75	1.13
CPn1011	2			1.54	0.73	0.72	0.42	0.46	0	-0.18	0.22
CPn0960CP0900	2	UP		2.53	1.73	1.42	0.96	0.35	0	0.38	0.47
CPn0976CP0880	2	UP		2.97	1.94	2.48	1.44	0.42	0	0.92	0.84
CPn0997CP0858mesJ	2	UP		0.96	0.9	0.91	0.38	-0.14	0	0.5	0.03
CPn1010CP0843	2			1.21	1.07	1.17	0.61	0.18	0	0.15	-0.12
CPn1012CP0841yzeB	2			1.64	0.82	0.72	0.58	-0.12	0	-0.03	-0.19
CPn0944CP0915	2			2.76	2.8	1.85	0.97	0.22	0	0.33	1.06
CPn1018CP0835	2	UP		2.52	1.49	1.56	1.04	0.48	0	0.63	0.67
CPn1027CP0825	2			2.63	1.79	1.31	1.33	0.41	0	0.45	0.29
CPn1035CP0817aroE	2			1.32	-0.01	0.76	0.51	-0.13	0	0.51	0.17
CPn1045CP0807	2			1.12	0.63	0.68	0.49	0.3	0	0.02	-0.13
CPn0958CP0902plsB	2			2.48	1.18	0.89	0.88	0.45	0	-0.01	-0.02
CPn0929CP0937	2	DOWN		3.43	2.42	0.78	0.46	0.11	0	0.31	0.84
CPn0943CP0917	2			2.83	1.82	1.15	0.16	-0.08	0	1.05	0.97
CPn0772CP1100uvrD	2			0.95	0.51	0.76	0.16	-0.08	0	-0.02	-0.09
CPn0831CP1039	2	UP		2.07	1.01	0.82	0.51	-0.2	0	0.37	-0.27
CPn0783CP1089	2	UP		2.01	1.35	1.7	1.08	0.51	0	0.69	0.36
CPn0788CP1084sdhC	2	UP		2.26	1.29	1.01	0.85	0.04	0	0.13	-0.02
CPn0790CP1082sdhB	2	UP		2.1	0.91	1.28	0.52	0.47	0	-0.05	0
CPn0823CP1048yscT	2			2.26	1.72	0.99	0.52	0.15	0	0.36	0.17
CPn0837CP1032nth	2	UP		2.38	2.05	1.31	0.86	0.56	0	0.62	0.25
CPn0928CP0938	2			2.57	1.95	1.46	0.86	0.01	0	0.1	0.13
CPn0838CP1031thdF	2			1.47	1.05	0.83	0.74	0.16	0	0.1	-0.48
CPn0843CP1026	2			2.42	0.83	0.75	0.55	0.3	0	0.67	0.39
CPn0882CP0984	2			1.8	0.51	0.77	0.41	0.16	0	0.62	-0.05
CPn0898CP0968groEL3	2			1.65	0.8	0.79	0.72	-0.18	0	0.46	0.38
CPn0505CP0248	2			1.77	1.03	1.12	0.85	0.05	0	0.32	-0.12
CPn0561CP0189euo	2			2.43	2	1.42	0.61	-0.09	0	0.47	1.44

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0492CP0262	2	UP		3.32	2.21	1.74	1.26	0.34	0	0.66	0.35
CPn0486CP0268	2	UP		2.07	1.29	1.33	0.76	0.39	0	0.3	0.58
CPn0313CP0445acpS	2			1.11	0.59	0.79	0.25	0.33	0	0.09	-0.1
CPn0281CP0477dhnA	2	UP		2.96	1.67	1.35	0.16	0.28	0	0.64	-0.02
CPn0284CP0474	2			1.5	0.96	1.05	0.89	0.4	0	-0.09	-0.06
CPn0285CP0473	2			2.06	1.24	1.07	0.55	0.17	0	0.2	0.48
CPn0287CP0471	2			1.12	0.74	0.45	0.57	-0.19	0	-0.1	0.35
CPn0288CP0470	2	UP		1.17	0.57	1.07	0.57	0.23	0	0.62	0.52
CPn0320CP0437ribF	2			1.08	1.28	0.85	0.87	0.07	0	0.43	-0.33
CPn0268CP0492	2	UP		3.17	2.1	1.9	1.08	-0.01	0	0.55	0.64
CPn0328CP0429	2	UP		1.81	0.52	1.07	0.6	0.15	0	0.31	0.34
CPn0336CP0422yojL	2			1.74	0.73	0.75	0.66	0.15	0	0.45	0.21
CPn0353CP0406	2	UP		1.92	0.79	1.47	0.73	0.35	0	0.1	0.17
CPn0354CP0405	2			0.56	0.22	0.64	-0.08	0.05	0	0.28	0.05
CPn0366CP0391	2	UP		2.96	1.67	1.04	0.95	-0.29	0	0.63	0.97
CPn0272CP0487dnaX2	2			1.05	0.78	0.49	0.59	0.13	0	-0.34	0.07
CPn0267CP0493	2			3.04	2.07	1.64	0.89	0.31	0	0.47	0.19
CPN0368_CP0389	2			1.52	0.93	0.42	0.49	0.08	0	0.45	0.4
CPn0224CP0541	2			2.48	1.5	0.48	0.63	0.24	0	0.5	1
CPn0201CP0566oppD	2			1.38	0.25	0.79	0.47	0.32	0	0.02	0.54
CPn0212CP0553	2	UP		3.22	2.14	1.61	0.76	0.55	0	0.58	0.98
CPn0218CP0547	2	UP		2.87	2.18	1.06	1.38	0.1	0	0.7	0.46
CPn0219CP0546tgt	2			0.93	0.68	0.78	0.75	0.01	0	0.13	0.3
CPn0223CP0542	2			2.32	1.32	1.16	1.11	0.03	0	0.3	0.28
CPn0225CP0539	2	UP		2.06	1.3	0.71	0.99	-0.1	0	0.26	0.55
CPn0266CP0495	2	UP		3.79	2.88	1.91	1.59	0.33	0	0.69	0.77
CPn0226CP0538	2			1.73	0.7	0.04	0.68	-0.18	0	0.63	0.26
CPn0240CP0522	2	UP		2.53	1.86	1.78	0.89	0.59	0	0.2	0.72
CPn0241CP0521	2			1.98	0.82	0.78	0.84	0.52	0	0.3	0.51
CPn0242CP0520	2			2.61	1.42	0.42	0.09	0.26	0	1.06	1.3
CPn0261CP0500ydaO	2	UP		1.5	0.75	1.22	0.94	-0.23	0	-0.34	-0.11
CPn0367CP0390	2			1.48	0.6	0.43	0.72	-0.05	0	0.05	0.17
CPn0348CP0412ytgB1	2	UP		1.97	0.93	1.16	0.36	0.27	0	0.72	0.12
CPj0339Cpn0339	2			1.46	0.71	0.62	0.65	0.46	0	0.55	0.66
CPn0452CP0301pmp12	2			1.68	1.37	1.08	0.5	-0.29	0	-0.15	0.23
CPn0431CP0322	2			1.02	0.69	0.74	0.26	0.28	0	0.49	0
CPn0432CP0321	2			1.36	0.82	0.48	0.34	-0.14	0	-0.11	-0.14
CPn0436CP0317	2		Yes	1.81	0.85	0.38	0.57	-0.05	0	0.34	0.33
CPn0439CP0314	2			1.72	1.43	0.96	0.79	0.42	0	-0.66	0.02
CPn0442CP0311	2			2.36	1.22	1.46	0.85	0.6	0	0	0.19
CPn0461CP0291	2			1.26	0.53	1.14	0.42	0.03	0	0.46	0.27
CPn0406CP0349	2		Yes	0.75	0.25	0.55	0.14	0.3	0	-0.06	0.53
CPn0464CP0288	2	UP		3.09	2.41	1.69	0.93	0.21	0	0.53	0.77
CPn0465CP0287	2	UP		2.62	1.94	1.82	0.95	0.21	0	0.41	0.63
CPn0470pmp173	2			1.9	0.93	1.09	0.75	-0.19	0	0.11	0.54
CPn0477CP0277yqeV	2			1.37	1.05	1.07	0.7	0.45	0	0.11	0.01
CPn0481	2			0.66	0.55	0.15	0.02	0.34	0	0.11	-0.08
CPn0429CP0324nqr4	2			1.85	0.93	0.47	0.59	0.14	0	0.15	0.02
CPn0435CP0318	2			0.64	0.43	0.23	-0.09	0.13	0	0.05	-0.13
CPn0380CP0376hemN1	2	UP		2.83	1.78	1.81	1.11	-0.04	0	0.89	0.6
CPn0372CP0385	2			1.62	1.43	0.85	0.37	0.5	0	0.04	0.08
CPn0400CP0355	2			1.81	0.66	0.75	0.67	0.07	0	0.57	0.38
CPn0404CP0351	2	UP		2.24	1.52	1.3	0.87	-0.46	0	0.68	0.79
CPn0737CP0008recC	1			0.85	0.22	-0.11	0.12	0.19	0	0.32	-0.5
CPn1072CP0777	1			1.12	-0.09	0.39	-0.2	0.26	0	0.41	0.12
CPn0475CP0279glgB	1			0.72	0.08	0.3	0.47	-0.17	0	-0.14	0.09
CPn0751CP1121	1			1.07	-0.09	-0.19	0.49	0.76	0	0.64	-0.14
CPn0957CP0903ide	1			0.58	-0.16	0.32	0.27	0.46	0	0.05	-0.01
CPn0980CP0876	1			0.14	-0.26	-0.02	0.04	0.12	0	0.16	-0.24
CPn0480CP0274	1			1.06	0.5	0.27	0.45	0.2	0	0.24	-0.23
CPn0526CP0226kpsF	1			1.02	0.33	0	0.42	-0.17	0	-0.19	-0.23
CPn0654CP0093yciA	1			1.45	0.44	0.37	0.47	-0.24	0	0.28	-0.08
CPn0763CP1109ygfA	1			2.01	0.56	0.6	0.48	0.51	0	0.17	-0.31
CPn0516CP0237	1			1.99	0.72	0.17	0.17	0.15	0	0.12	-0.19
CPn0514CP0239	1			1.12	-0.14	0.27	0.44	0.41	0	0.06	-0.04
CPj0820CPn0820	1			0.73	-0.2	0.22	0.3	0.55	0	-0.04	0.15
CPn0483CP0271	1			0.69	0.04	0.4	0.2	0.58	0	0.37	0.08
CPn0947CP0912pgsA2	1	UP		1.6	0.67	0.68	0.57	0.38	0	-0.05	-0.12
CPn0815CP1056gspD	1		Yes	1.11	0.26	0.25	0.18	0.54	0	0.12	0.12
CPn0764CP1108	1			1.61	0.53	-0.07	0.1	-0.04	0	0.21	-0.26
CPn0765CP1107	1			1.76	0.13	0.18	0.22	0.55	0	0.11	-0.15
CPn0814CP1057	1			1.34	0	0.29	0.53	0.01	0	0.34	0.47
CPn0830CP1041	1	UP		0.48	-0.33	0.28	0.42	0.3	0	-0.07	-0.34
CPn0819CP1052	1			0.69	-0.18	0.15	0.36	0.28	0	0.15	-0.09
CPn0821CP1050	1			0.6	0.25	0.23	0.25	0.38	0	-0.05	-0.47

NAME	Cluster	Persistency	in EB	6 h	12 h	18 h	24 h	36 h	48 h	60 h	72 h
CPn0662CP0085aspS	1		Yes	1.16	0.64	0.26	0.43	0.39	0	-0.15	-0.2
CPn0817CP1054gspF	1			0.79	-0.12	0.14	0.3	0.57	0	-0.13	-0.22
CPn0784CP1088exbD	1			1.37	0.1	0.29	0.56	-0.32	0	0.25	-0.37
CPn0844CP1025yphC	1			1.04	0.31	0.36	0.29	0.61	0	0	-0.17
CPn0816CP1055gspE	1			1.78	0.52	0.75	0.71	0.23	0	-0.02	0.04
CPn0381CP0375	1			1.17	0.54	0.63	0.77	0.27	0	-0.07	-0.39
CPn0484CP0270aroG	1			0.78	0.31	0.15	0.37	-0.18	0	-0.13	-0.36
CPn0868CP1001zntA	1			2.53	1.12	0.64	0.34	0.17	0	-0.05	-0.18
CPn0878CP0991	1			0.53	0.19	0.27	0.29	0.18	0	-0.09	-0.33
CPn0862CP1007yfhO2	1			2.07	0.34	0.36	0.32	0.06	0	0.08	-0.11
CPn0031CP0745rs21	1			1.19	0.82	0.08	0.13	0.02	0	-0.07	-0.24
CPN03681_CP03891	1	DOWN		1.26	0.69	0.14	0.48	0	0	-0.1	-0.05

Supplementary-Table 2 – **Detected mRNA transcripts present in the infectious EB form.**

Cpn1011	*Cpn0971yccA2*	*Cpn0970tyrP2*	*Cpn969.1yccA1*	*Cpn0958*
Cpn0947pgsA2	*Cpn0943*	*Cpn0933*	*Cpn0913*	*Cpn0886*
Cpn0876dagA2	*Cpn0843*	*Cpn0808*	*Cpn0796*	*Cpn0790*
Cpn0788	*Cpn0715*	*Cpn0701*	*Cpn0695*	*Cpn0682*
Cpn0679	*Cpn0678*	*Cpn0677*	*Cpn0661mip*	*Cpn0656*
Cpn0644	*Cpn0641*	*Cpn0640*	*Cpn0572*	*Cpn0560*
Cpn0559	*Cpn0558*	*Cpn0557omcB*	*Cpn0538*	*Cpn0523*
Cpn0473	*Cpn0451pmp11*	*Cpn446pmp8*	*Cpn429nqr4*	*Cpn416himD*
Cpn0384hctB	*Cpn0351adt1*	*Cpn0333*	*Cpn0332*	*Cpn0268*
Cpn0266	*Cpn0212*	*Cpn0168*	*Cpn0135groES*	*Cpn0132groEL*
Cpn0028	*Cpn0026*	*Cpn0001*		

Supplementary-Table 3 – Significantly regulated genes of the iron-depletion mediated persistent infection and iron repletion. The columns are name (*Cpn* for sequences specific for strain *CWL029* and Cp for sequences specific for strainAR39), Cluster (according to Figure 19) and time points from 24 h p.i. to 72 h p.i. and 96 h p.i. to 120 h p.i.(data in log2).

NAME	Cluster in acute infection	DAM 24 h p.i.	DAM 48 h p.i.	DAM 72 h p. i.	Repleted 96 h p.i.	120 h p.i.
CPn1073CP0776	2	0.4	1.1	0.96	2.62	1.62
CPn1072CP0777	1	0.26	0.55	0.77	1.89	0.95
CPn1069CP0781yfgA	10	0.05	0.13	0.36	-0.26	0.28
CPn1067CP0783def		0.13	0.67	0.74	0.13	0.24
CPn1066CP0784		-0.08	0.85	0.9	-0.43	0.02
CPn1065	3	0.1	1.15	1.03	0.72	1.2
CPn1064CP0785		0.1	0.2	0.83	-0.33	-0.05
CPn1063CP0786tpiS	2	0.04	0.87	1.06	0.66	0.31
CPn1060CP0790tktB2	6	-0.06	-0.33	-0.84	-0.63	-0.69
CPn1059CP0791kgsA	4	0.16	-0.24	-0.41	-0.42	-0.62
CPn1058CP0792		0.01	0.77	0.7	-0.51	-0.42
CPn1057CP0793		0	0.1	-1.55	1.26	0.65
CPn1055CP0795		0.14	0.58	0.7	0.69	0.37
CPn1053CP0799	3	-0.05	0.25	0.73	0.8	0.42
CPn1052CP0800	3	0.22	0.93	0.06	0.23	0.33
CPn1050CP0802dapA		-0.03	0.61	0.74	-0.21	-0.15
CPn1049CP0803lysC	7	-0.1	-0.79	-0.68	-0.51	-0.5
CPn1039CP0813aroA		-0.19	0.85	0.54	-0.46	-0.76
CPn1038CP0814aroL		-0.16	0.62	0.92	0.04	0.13
CPn1037CP0815aroC		0.05	0.38	0.67	0.03	0.17
CPn1036CP0816aroB		0.18	-0.02	0.16	-0.18	-0.9
CPn1035CP0817aroE	2	-0.13	0.31	-0.05	2.04	0.31
CPn1034CP0818	10	0.03	0.14	0.49	0.42	0.06
CPn1033CP0819	10	-0.5	-1.28	-0.9	-1.21	-0.07
CPn1032CP0820	9	-0.48	-0.94	-0.43	-0.23	-0.17
CPn1031CP0821arcD	7	0.06	-0.92	-0.18	-0.8	0.16
CPn1030CP0822		-0.07	0.49	0.76	0.72	0.12
CPn1029CP0823	8	-0.28	0.45	0.59	-0.46	-0.67
CPn1027CP0825	2	0.33	0.7	-0.08	1.53	0.78
CPn1025CP0827pgi	12	0.1	-0.15	-1.05	0.25	0.69
CPn1024CP0828xerD		0.27	0.58	0.56	0.34	0.14
CPn1023CP0829	12	0.08	-0.9	-1.55	0.56	0.83
CPn1018CP0835	2	0.45	2.12	1.63	0.57	2.14
CPn1017CP0836lytB	6	0.36	-0.22	0.02	-0.5	-0.37
CPn1015CP0838	5	0.22	0.64	1.16	-0.25	-0.55
CPn1013CP0840fumC	9	-0.58	-0.71	-0.38	-0.59	-0.54
CPn1011	2	0.08	-0.18	-0.82	-0.54	0.6
CPn1007CP0846		-0.19	-0.02	0.14	0.5	0.18
CPn1006CP0847	12	-0.39	-1.5	-1.03	-0.9	1.28
CPn1005CP0848	11	-0.36	-1.52	-1.69	-0.29	0.77
CPn1004CP0849	11	-0.3	-1.84	-1.52	-0.53	0.5
CPn1001CP0852yfhC	8	0.17	0.83	0.96	-0.04	0.54
CPn0999CP0855pnp		0.04	0.45	0.74	1.31	0.58
CPn0998CP0857ftsH	10	-0.14	-0.78	-0.82	-0.09	0.1
CPn0997CP0858mesJ	2	-0.02	1.07	0.98	1.09	0.25
CPn0995CP0860		-0.1	-0.06	-0.2	-0.56	-0.94
CPn0994CP0861	11	0.14	-1.09	-1.56	-0.22	-0.57
CPn0990CP0865infC	6	-0.01	1.26	1.02	-0.5	-0.33
CPn0988CP0867murB		0.02	0.05	-0.05	-0.3	-0.13
CPn0986CP0869yggH	3	0.24	0.93	0.84	0.05	0.01
CPn0985CP0871nrdB	7	-0.24	-0.96	-1	-0.91	-0.43
CPn0984CP0872nrdA	7	0.03	-1.2	-1.04	-0.81	-0.08
CPn0981CP0875		0.31	0.85	0.87	-0.3	0.81
CPn0980CP0876	1	0.06	0.3	0.59	0.7	0.04
CPn0979CP0877htrA	2	-0.25	1.25	1.16	-0.13	0.69
CPn0976CP0880	2	0.47	1.53	1.04	1.95	2.66
CPn0971CP0888yccA2	10	0.1	0.65	0.65	0.41	0.67
CPn09691yccA1	10	-0.18	0.08	0.94	0.44	0.37
CPn0966CP0894pcnB	5	0.25	-0.59	-0.63	0.03	-0.49
CPn0961CP0899rl32	4	-0.04	0.79	1.18	0.33	0.12
CPn0960CP0900	2	0.67	2.07	1.77	-0.29	1.92
CPn0956CP0904	10	-0.6	-1.03	-0.81	-0.71	-0.62
CPn0948CP0911glgA		0.46	1.15	0.62	-0.82	0.95
CPn0947CP0912pgsA2	1	0.22	1.18	0.77	1.8	0.53

NAME	Cluster in acute infection	DAM 24 h p.i.	DAM 48 h p.i.	DAM 72 h p. i.	Repleted 96 h p.i.	Repleted 120 h p.i.
CPn0938CP0923		-0.11	0.46	1.17	-0.46	-0.82
CPn0936CP0925rl36	9	0.18	0.39	0.27	-0.93	0.62
CPn0935CP0926rl34		0.43	0.05	-0.02	0.26	0.27
CPn0934CP0927rnpA	3	0.57	-0.64	-0.71	-0.2	0.02
CPn0930CP0935	12	0.32	-0.46	-1	0.57	0.42
CPn0929CP0937	2	-0.12	-0.43	-1.35	0.58	0.45
CPn0927CP0939	3	-0.28	0.35	0.4	0.9	0.37
CPn0921CP0945	8	-0.32	-0.5	-0.45	-0.81	-0.53
CPn0913CP0953	9	0.52	-0.38	-0.69	-1.17	-0.28
CPn0912CP0954	6	0.6	0.07	-0.3	-1.2	-0.51
CPn0909CP0957rsbV2	8	-0.06	0.06	0.85	-0.46	-0.21
CPn0902CP0964nlpD	9	0.24	0.99	1.54	0.83	-0.03
CPn0897CP0969		-0.19	-0.26	0.02	0.15	0.19
CPn0895CP0971efp2		-0.39	0.29	0.9	-0.56	-0.27
CPn0894CP0972amn	9	-0.15	-1.26	-0.82	-0.13	-0.61
CPn0891CP0975mfd	6	-0.29	0.6	0.74	0.57	-0.14
CPn0888CP0978hemG		0.15	0.3	0.04	-0.9	-0.99
CPn0887CP0979		-0.18	0.69	0.72	0.94	0.52
CPn0884CP0982	8	-0.17	-1.03	0.02	-1.57	-0.32
CPn0882CP0984	2	-0.21	0.1	0.59	-0.09	0.23
CPn0879CP0990yycJ		-0.12	-0.9	-0.72	-0.59	-0.78
CPn0873CP0996ribE	8	-0.07	0.15	0.14	-0.88	0.2
CPn0871CP0998ribD	3	0.12	0.57	0.86	0.13	0.2
CPn0870CP0999serS	7	-0.06	-0.61	-0.87	-1.12	-0.29
CPn0869CP1000		0.18	-0.45	-0.29	-1.12	-0.56
CPn0867CP1002rodA	3	0.42	1.25	1.09	1.35	0.48
CPn0866CP1003birA		0.01	0.64	0.8	-0.07	-0.35
CPn0862CP1007yfhO2	1	0.25	0.61	0.78	2.53	0.28
CPn0860CP1009fliF	10	-0.7	-1.8	-0.95	-1.26	-0.23
CPn0859CP1010		0.31	-0.03	-0.19	1.2	0.28
CPn0857CP1012	10	-0.19	-1.74	-0.81	-1.48	-0.37
CPn0853CP1016	11	-0.08	-2.01	-1.81	-0.56	0.26
CPn0852CP1017	11	-0.29	-2.49	-2.29	-0.66	0.18
CPn0849CP1020	8	-0.17	-1	-0.91	-1.35	-0.61
CPn0848CP1021tigA	4	-0.12	-0.37	-0.73	-1.02	-0.23
CPn0844CP1025yphC	1	0.34	0.65	0.31	-0.85	-0.66
CPn0843CP1026	2	0.46	0.7	0.42	1.32	0.98
CPn0842		-0.2	0.13	-0.1	1.69	0.73
CPn0841CP1028secA2		0.13	-1	-0.87	-0.83	-0.35
CPn0837CP1032nth	2	0.8	2.17	1.62	3.58	2.09
CPn0831CP1039	2	0.32	0.76	1	1.78	0.49
CPn0830CP1041	1	0.08	0.89	1.36	0.77	-0.11
CPn0829CP1042	4	0.12	0.82	0.91	0.52	0.33
CPn0826CP1045yscL	7	0.16	-1.03	-0.76	-1.12	-0.56
CPn0824CP1047yscS	10	-0.21	-0.61	-0.76	-1.3	-0.5
CPn0822CP1049	10	-0.26	-0.56	-0.31	0.24	-0.04
CPn0818CP1053		0.14	-0.51	-0.67	-0.57	-0.44
CPn0817CP1054gspF	1	0.3	-0.44	-0.72	-0.06	-0.95
CPn0813CP1058pepP	2	0.25	0.32	0.45	-0.35	-0.17
CPn0811CP1060lcrH1	11	-1.03	-3.11	-1.32	-0.44	1.41
CPn0810CP1061	11	-0.63	-2.31	-1.84	-0.49	1.21
CPn0809CP1062	11	-0.3	-1.89	-0.64	0.07	1.51
CPn0808CP1063	10	-0.32	-2.25	-1.94	-0.06	1.38
CPn0807CP1064		0.32	1.18	1.09	0.06	0.13
CPn0806CP1065thrS	7	-0.05	0.47	0.95	-0.79	-0.44
CPn0801CP1070	4	-0.45	-0.76	-0.84	-0.62	-0.24
CPn0798CP1073	9	-0.22	0.9	1	0.14	-0.47
CPn0794CP1077	11	-0.39	-1.61	-0.91	-0.87	-0.18
CPn0791CP1081	11	-0.18	-1.01	-1.19	-0.45	0.08
CPn0790CP1082sdhB	2	-0.04	1	0.35	1.57	0.16
CPn0788CP1084sdhC	2	0.53	1.37	0.8	3.15	0.86
CPn0787CP1085yabD	3	0.15	1.3	0.94	0.48	0.04
CPn0783CP1089	2	0.35	1.68	1.29	0.94	1.05
CPn0780CP1092amiB		-0.39	-0.67	-0.32	-0.78	-0.92
CPn0779CP1093		-0.19	-0.59	0.29	0.36	-0.2
CPn0778CP1094ahpC	7	-0.47	0.46	0.88	-0.5	-0.38
CPn0777CP1095groEL2	10	0.1	-1.11	-1.05	-0.21	-0.63
CPn0773CP1099ung		0.3	0.33	0.4	0.54	-0.48
CPn0769CP1103topA	10	0.09	-0.94	-0.7	-1.45	-0.21
CPn0767CP1105		-0.35	0.7	0.8	0.34	-0.3
CPn0755CP1117		0.1	-0.41	-0.13	1.57	0.06
CPn0753CP1119	6	0.02	1.08	0.91	-0.7	-0.07
CPn0750CP1122tctD	9	-0.72	-1.7	-1.36	-0.48	-0.26
CPn0742CP0003	10	-0.37	-0.97	-0.64	0.37	0.97

NAME	Cluster in acute infection	DAM 24 h p.i.	DAM 48 h p.i.	DAM 72 h p. i.	Repleted 96 h p.i.	120 h p.i.
CPn0740tyrB	6	0.02	-0.02	-0.4	-0.58	-0.85
CPn0738CP0007	6	-0.32	-0.43	-0.56	-0.13	-0.68
CPn0734yceA	5	0.11	0.11	-0.06	-0.38	-0.46
CPn0732nfo		0.48	0.53	0.73	1.06	1.15
CPn0731CP0015	10	-0.18	-0.42	-0.42	0.61	0.44
CPn0730CP0016mviN	2	0.62	1.32	0.53	1.81	0.18
CPn0728CP0018	11	0.06	-1.88	-2.34	0.24	1.37
CPn0727CP0019	11	-0.03	-2.09	-2.41	-0.46	0.56
CPn0726	12	0.05	-0.77	-0.75	0.2	0.7
CPn0725	11	-0.47	-1.52	-1.59	0.14	0.39
CPn0724CP0022	12	0.02	-0.64	-1.06	0.23	0.21
CPn0723yhbG	3	0.18	0.69	1.07	2.05	1.17
CPn0720CP0026	11	-0.66	-0.48	-1.32	-0.04	1.19
CPn0718CP0028		0.1	-0.74	-0.53	0.11	-0.1
CPn0716CP0030	6	-0.36	-0.85	-0.59	-0.52	-0.52
CPn0712CP0034	8	-0.01	-1.35	-1.01	-0.95	-0.55
CPn0711CP0035	7	-0.41	-1.39	-0.78	-1.05	-0.33
CPn0708CP0038	8	-0.11	-1.35	-0.87	-0.79	-0.2
CPn0707CP0039yscN	7	-0.27	-1.16	-0.96	-1.2	-0.28
CPn0706CP0040		-0.47	-0.69	-1.1	-0.12	-0.81
CPn0705CP0041	7	-0.45	-1.43	-1.17	-0.82	-0.78
CPn0704fliN	8	-0.26	-0.82	-0.8	-0.83	-0.8
CPn0702CP0044yscC	7	-0.5	-1.61	-0.95	-1.03	-0.37
CPn0701CP0045karG	10	0.34	-1.06	-0.81	-1.07	0.11
CPn0700CP0046	11	-0.71	-1.12	-0.73	-0.59	0
CPn0697CP0049tsf		0.42	0.04	-0.09	-1.28	0.03
CPn0696rs2		0.25	0.32	0.24	-0.75	0.03
CPn0693	11	-0.25	-1.24	-1.19	-0.33	0.05
CPn0688CP0058	5	0.07	-0.82	-0.7	-0.58	-0.87
CPn0685CP0061		1.24	-0.34	0.47	-0.38	0.46
CPn0682CP0065dppD	2	0.51	1.38	1.26	2.78	1.31
CPn0679CP0068	1	0.36	1.52	1.23	2.43	1.18
CPn0678	12	0.01	-0.72	-0.72	1.12	2.46
CPn0677CP0070	10	-0.28	-2.2	-1.83	0.13	1.63
CPn0676CP0071	12	-0.01	-2.3	-2.46	-0.05	1.56
CPn0675CP0072		-0.14	-0.88	-1.85	0.44	0.08
CPn0670rsbW	7	-0.25	-1.26	-0.91	-1.12	0
CPn0669CP0078	10	-0.45	-1.35	-0.84	-0.92	0.2
CPn0664		0.08	1.02	1.02	0.65	0.89
CPn0660spoU2		-0.13	-0.4	-0.14	-0.96	-0.61
CPn0658	5	-0.18	0.28	0.78	0.02	-0.18
CPn0657CP0090		-0.11	-0.29	-0.3	-0.72	-0.02
CPn0655CP0092dnaQ2	4	-0.12	-0.47	-0.94	-1.17	-0.2
CPn0654CP0093yciA	1	0.18	0.78	0.8	0.9	0
CPn0651CP0096fabZ		0.11	-0.51	-0.68	-0.9	-0.12
CPn0650CP0097lpxA	6	-0.11	-0.21	-0.59	-0.84	-0.36
CPn0649CP0098fmt	4	0.04	-1.02	-0.92	-0.72	0.31
CPn0635CP0112rl5	5	0.14	-0.21	-0.72	-1.38	-0.48
CPn0634CP0113rs8	5	-0.08	-0.21	-0.85	-1.44	-0.63
CPn0633CP0114rl6	5	-0.11	-0.6	-0.91	-1.26	-0.29
CPn0632CP0115rl18	5	0.21	-0.54	-0.87	-1.31	-0.49
CPn0631CP0116rs5	6	0.2	-0.6	-0.98	-1.38	-0.27
CPn0630CP0117rl15	5	-0.06	-0.47	-0.78	-1.29	-0.45
CPn0629CP0118secY	4	0.34	-0.49	-0.85	-1.53	-0.15
CPn0626CP0121rpoA	5	-0.2	-0.34	-0.64	-1.53	-0.73
CPn0620CP0127ruvA	2	0.16	1.05	0.88	2.13	0.98
CPn0618CP0129lplA2	2	-0.17	1.1	0.95	2.7	1.44
CPn0613CP0134sohB	6	0.11	0.29	0.71	-0.01	-0.3
CPn0611CP0136yacE	4	-0.15	-0.62	-0.74	-0.27	-0.37
CPn0610CP0137rho		-0.5	-0.32	-0.9	-0.39	-0.01
CPn0607CP0140glgC	12	0.06	-1.14	-1.21	0.77	-0.25
CPn0596CP0152ada	2	0.45	0.7	0.49	1.82	0.82
CPn0589CP0159	3	0.02	-0.7	-1.03	-0.76	-0.08
CPn0588CP0160	2	0.47	1.1	-0.21	2.96	1.67
CPn0587CP0161yvyD	11	-0.28	-0.48	-0.72	0.12	0.54
CPn0586CP0162atoC	12	0.06	0.16	0.2	2.11	0.68
CPn0584CP0164atoS	11	-0.36	-1.46	-0.96	-0.97	-0.16
CPn0583CP0165		-0.22	-0.24	-0.69	-0.51	0.3
CPn0580CP0168truA	2	0.14	0.99	0.8	2.03	0.72
CPn0579CP0169yacM	2	0.41	0.35	0.12	1.4	1.31
CPn0578CP0170yaeI	4	0.07	-0.04	-0.24	0.05	-0.24
CPn0577CP0171	9	0.3	0.06	0.19	-1.32	-0.11
CPn0575aprfB	5	0.16	-0.09	0.27	-0.23	-0.32
CPn0572CP0177	1	0.3	0.47	-0.97	2.34	1.35

NAME	Cluster in acute infection	DAM 24 h p.i.	DAM 48 h p.i.	DAM 72 h p. i.	Repleted 96 h p.i.	120 h p.i.
CPn0568CP0181		0.07	-0.65	-0.64	0.78	-0.57
CPn0567CP0182cdsA		-0.03	-0.55	-0.66	-1.1	-0.77
CPn0565CP0185	2	0.38	0.38	-0.03	0.21	0.61
CPn0559CP0191	12	0.28	0.01	-0.89	1.79	2.18
CPn0558CP0193	11	-1.36	-1.18	0.01	-0.29	1.78
CPn0557CP0195omcB	10	-1.85	-2.27	-0.44	-0.51	1.18
CPn0555CP0197tsp		-0.13	-1.24	-1.09	0.33	0.17
CPn0554CP0198	3	0.18	1.29	1.03	1.61	0.57
CPn0550fusA	4	-0.27	-0.3	-0.61	-1.32	-0.56
Cpn0548_CP0204	5	-0.1	-0.09	-0.64	-1.04	-1.27
CPn0545CP0207rl27		-0.2	0.61	0.97	-0.1	0.67
CPn0542CP0210	4	0.28	0.56	0.06	0.49	-0.2
CPn0539CP0213pmp19	5	0.07	0.68	0.85	-0.08	-0.59
CPn0538CP0214	11	-1.11	-1.72	-1.8	0.25	1.32
CPn0537CP0215	12	-0.47	-1.66	-1.63	1.32	1.66
CPn0535CP0217lspA	8	0.4	-0.24	-0.79	-1.47	-0.5
CPn0529CP0223ycaH	2	0.03	0.7	0.78	0.31	-0.04
CPn0528CP0224gltT		0.17	0.41	0.81	-0.55	0.09
CPn0524CP0229	12	-0.37	-1.11	-1.88	-0.31	0.96
CPn0522CP0231	2	0.21	1.01	1.04	2.48	1.01
CPn0517CP0236		0.09	0.75	-0.13	2.15	0.55
CPn0515CP0238ubiE	2	0.35	0.85	0.6	1.28	-0.18
CPn0514CP0239	1	0.3	0.26	-0.05	0.07	-0.15
CPn0513CP0240		0.2	0.38	0.11	0.45	-0.41
CPn0504CP0249vacB	5	-0.34	-0.33	-0.56	-1.24	-0.64
CPn0503CP0251dnaK		-0.48	-0.87	-0.25	-0.5	0.23
CPn0502CP0252grpE		0.24	-0.2	-0.11	-0.65	0.03
CPn0499CP0255	2	0.36	1.26	-0.05	3.11	2.06
CPn0496CP0258		0.27	0.28	0.18	-0.27	-0.2
CPn0495CP0259aspC	8	0.58	0.52	0.4	-0.54	0.08
CPn0494CP0260	12	0.06	-1.12	-1.23	0.85	1.51
CPn0493CP0261	12	0.17	0.22	-0.82	1.61	1.41
CPn0492CP0262	2	0.49	1.66	1.23	2.77	1.83
CPn0487CP0267	6	0.2	-0.2	-0.85	0.13	-0.85
CPn0486CP0268	2	0.39	1.15	0.78	2.63	1.44
CPn0473CP0281	12	0.29	1.18	0.15	1.86	2.24
CPn0472CP0282	11	-0.23	-1.16	-1.92	-0.35	1.16
CPn0470pmp173	2	0.08	0.04	0.03	1.11	0.63
CPn0467CP0285pmp16	10	0.07	-1.11	-1.03	-1.05	-0.05
CPn0466CP0286pmp15	11	-0.09	-0.37	-0.64	0.46	0.54
CPn0465CP0287	2	0.41	1.54	1.4	1.63	1.37
CPn0464CP0288	2	0.3	1.58	1.09	2.61	0.94
CPn0463CP0289	3	0.01	0.59	0.52	2.41	0.59
CPn0461CP0291	2	0.13	0.76	0.38	0.79	0.76
CPn0459CP0293		-0.15	-0.01	-0.34	1.32	1.2
CPn0454CP0298pmp14		0.12	-0.25	0.02	1.46	0.73
CPn0453CP0299pmp13	9	-0.56	-1.16	-0.84	-1.74	-0.33
CPn0448CP0305yxjG2	7	-0.44	0.02	0.14	-0.39	-0.74
CPn0446CP0307pmp8	9	-0.37	-1.77	-1.53	-0.8	-0.09
CPn0445CP0308pmp7	10	-0.26	-0.96	-0.73	-0.23	0.17
CPn0444CP0309pmp6	9	-0.34	-1.05	-0.9	-0.39	-0.27
CPn0443CP0310		0.19	-0.16	-0.76	0.82	1.66
CPn0442CP0311	2	0.49	0.55	0.14	0.83	0.39
CPn0440CP0313	5	-0.16	0.35	-0.77	-0.68	-0.53
CPn0439CP0314	2	0.13	0.23	-0.25	-0.43	0.27
CPn0434CP0319	8	-0.18	-0.32	-0.25	-0.91	0.46
CPn0433CP0320gcsH	10	0.08	-0.34	-0.11	-0.96	-0.05
CPn0429CP0324nqr4	2	0.25	0.42	0.17	0.9	0.32
CPn0419CP0335pbp3	11	-0.01	-0.81	-0.56	-0.63	-0.48
CPn0418CP0336murE	3	0.23	1.26	1.08	-0.5	2.13
CPn0416CP0338himD	10	-0.85	-1.11	0.46	-0.44	0.11
CPn0415CP0339	8	-0.06	-0.52	-1.09	-1.24	-0.95
CPn0408CP0346		0.15	0.45	0.66	0.81	0.57
CPn0406CP0349	1	0.04	0.58	0.48	1.02	0.44
CPn0404CP0351	2	0.73	2.13	1.61	3.44	2.49
CPn0403yceC	3	0.1	1.11	1.31	1.97	1.16
CPn0402CP0353mutY	7	-0.14	-0.2	0.02	0.06	0.05
CPn0395CP0360		-0.62	-0.34	-0.58	-0.42	-0.38
CPn0391CP0364		-0.75	-0.05	0.17	0.28	0.09
CPn0390CP0365ruvB	8	-0.15	-0.45	-0.22	-0.34	0.19
CPn0389CP0366	8	-0.35	-0.2	0.08	-0.57	-0.52
CPn0386CP0369ssb		-0.15	-0.64	-0.5	-0.96	-0.34
CPn0385CP0370pepA		0.03	-0.29	-0.65	-0.83	-0.17
CPn0384CP0371hctB	11	-2.46	-2.97	-0.29	-0.81	1.14

NAME	Cluster in acute infection	DAM 24 h p.i.	DAM 48 h p.i.	DAM 72 h p. i.	Repleted 96 h p.i.	120 h p.i.
CPn0380CP0376hemN1	2	0.05	1.18	1.01	2.57	1.56
CPn0376CP0380		-0.51	0.11	0	0.81	0.77
CPn0375CP0381		-0.16	0.35	0.32	-0.69	0.27
CPn0370CP0387		0.19	-0.11	-0.39	0.67	0.61
CPn0369CP0388	12	-0.02	-1.04	-1.12	0.53	1.02
CPN03681_CP03891	1	-0.12	-0.82	-0.96	0.1	0.11
CPn0366CP0391	2	0.36	1.24	0.13	1.05	1.22
CPn0365CP0392	12	0.07	-0.69	-0.79	0.51	0.68
CPn0363CP0394flhA		-0.02	-0.83	-0.66	-0.63	-1.02
CPn0362CP0395rpsD	11	-0.47	-1.38	-1.25	0.03	0.99
CPn0358CP0400	3	0.03	0.06	0.99	-0.25	-0.39
CPn0357CP0401		-0.13	0.72	1.33	-0.04	-0.58
CPn0355CP0404	10	-0.37	0.26	0.74	-0.74	-0.02
CPn0353CP0406	2	0.14	1.75	1.43	0.41	2.14
CPn0348CP0412ytgB1	2	0.28	0.91	0.95	-0.06	0.55
CPn0340CP0417	12	0.05	-0.93	-1.33	0.44	0.87
CPn0337CP0421smpB	9	-0.08	0.34	0.93	0.27	0.4
CPn0335folD		-0.16	0.56	0.24	-0.59	-0.38
CPn0334CP0424		0.17	0.63	0.43	0.36	0.01
CPn0332	12	0.08	-0.59	-2.06	1.31	2.8
CPn0331CP0426		0.26	0.63	-1.27	1.66	1.9
CPn0330CP0427	12	0.15	-1.74	-2	0.04	1
CPn0328CP0429	2	0.48	1.4	1.11	1.18	1.29
CPn0327CP0430rl28	5	0.11	0.27	0.54	-0.61	0.48
CPn0326CP0431malQ		-0.08	-0.42	0.06	0.68	-0.04
CPn0325CP0432sycE	10	-0.39	-1.59	-1.4	-1.07	-0.73
CPn0324CP0433lcrE	10	-0.35	-1.15	-0.8	-0.48	-0.47
CPn0318CP0439rbfA	5	-0.04	-0.21	-0.88	-1.27	-0.89
CPn0317CP0440infB	5	0.01	-0.37	-0.86	-0.96	-0.92
CPn0310CP044860IM		-0.23	0.48	0.75	-0.27	-0.32
CPn0309CP0449dnaA1	7	-0.08	-0.23	-0.46	-4.83	-0.6
CPn0308CP0450		0.34	0.61	0.21	0.91	0.67
CPn0307CP0451glgP	10	-1.11	-1.2	-0.98	0.21	-0.05
CPn0303CP0455		-0.11	1	1.1	-0.1	0.83
CPn0293CP0465		0.03	0.25	0.7	-0.29	-0.21
CPn0292CP0466incC	3	0.37	0	-0.59	-1.18	-0.25
CPn0291CP0467incB		0.01	-0.19	-1.18	-1.5	-0.54
CPn0290CP0468	4	0.42	0.84	0.74	-0.3	-0.03
CPn0288CP0470	2	0.49	0.9	0.98	1.82	0.99
CPn0285CP0473	2	0.15	0.1	0.19	-0.56	0.08
CPn0283	5	0.21	-0.03	0.11	0.5	-0.16
CPn0282CP0476xasA	8	-0.56	-0.35	-0.46	-1.05	-0.49
CPn0281CP0477dhnA	2	0.42	0.85	0.63	2.21	0.05
CPn0280CP0478dppF1		0.29	1.12	1.04	-0.27	-0.02
CPn0279CP0479	5	0.47	1.53	1.23	-0.48	-0.13
CPn0277CP0481		0.12	1.16	0.98	0.51	0.74
CPn0271CP0488		-0.31	0.51	0.27	-0.83	-0.13
CPn0268CP0492	2	0.01	1.49	1.24	1.99	1.61
CPn0267CP0493	2	-0.35	0.05	-0.02	0.82	-0.34
CPn0266CP0495	2	0.09	1.49	1.08	2.12	1.99
CPn0264CP0497ubiD	3	0.13	1.26	1.02	0.88	0.37
CPn0263CP0498yqfU	7	0.13	1.01	0.77	-0.55	0.2
CPn0261CP0500ydaO	2	0.06	1.62	1.16	0.17	1.02
CPn0260CP0501secA1	8	-0.31	0.91	0.78	-0.03	0.01
CPn0257CP0504		0.29	0.19	0.25	0.96	-0.16
CPn0256CP0505	11	-0.41	0.58	0.62	-0.25	0.48
CPn0255CP0506		0.12	1.01	0.38	1.17	0.84
CPn0254CP0507	12	-0.01	-0.09	-0.19	0.77	0.04
CPn0250CP0511rl33	8	0.13	0.46	0.57	-0.44	1.19
CPn0246CP0516rs9		-0.05	0.42	0.49	-0.08	0
CPn0245CP0517ydhO		0.1	0.34	-0.12	-0.32	-0.18
CPn0244CP0518adk		0.42	0.86	0.64	-0.79	-0.01
CPn0241CP0521	2	0.21	0.58	-0.22	2.38	0.82
CPn0240CP0522	2	0.71	1.69	1.18	2.51	1.79
CPn0237CP0525yggF	4	-0.33	-0.67	-0.69	-0.67	-0.2
CPn0236CP0526pyrG	4	0.07	-0.37	-0.71	-0.66	-0.25
CPn0235CP0527kdsB	4	-0.12	0.19	0.16	-0.09	-0.18
CPn0234CP0529	12	0.08	-0.04	-1.35	1.7	1.23
CPn0233CP0531		-0.07	-0.36	-1.82	0.72	0.13
CPn0231CP0533tauB	9	0.11	1.28	0.69	0.03	-0.07
CPn0229CP0535	10	-0.19	-0.71	-0.56	-0.9	0.07
CPn0228CP0536dsbG	10	-0.18	-2.07	-1.71	-1.72	-0.07
CPn0227CP0537dsbB	10	-0.43	-0.92	-1.53	-1.26	-0.1
CPn0225CP0539	2	0.13	0.93	0.43	0.32	0.32

NAME	Cluster in acute infection	DAM 24 h p.i.	DAM 48 h p.i.	DAM 72 h p. i.	Repleted 96 h p.i.	120 h p.i.
CPn0223CP0542	2	0.32	0.81	0.07	1.95	0.6
CPn0220CP0545		-0.03	-0.14	-0.91	0.52	1.33
CPn0219CP0546tgt	2	0.11	0.41	0.21	0.13	0.12
CPn0218CP0547	2	0.43	1.6	1.16	3.8	1.49
CPn0213CP0552		0.57	-0.6	-1.21	-0.51	-0.38
CPn0212CP0553	2	0.39	2.04	1.55	3.48	1.98
CPn0211CP0554	3	0.33	1.27	0.92	1.61	0.24
CPn0209CP0557	12	0.03	0.17	0	0.43	0.22
CPn0204CP0563	11	-0.18	-0.58	-0.86	0.47	-0.6
CPn0203CP0564		-0.11	0.8	-0.58	0.25	-0.6
CPn0201CP0566oppD	2	-0.05	0.81	0.25	0.63	0.54
CPn0198CP0569oppA4	2	-0.08	0.21	0.04	0.5	0.47
CPn0197CP0570oppA3	2	-0.12	0.24	-0.33	0.03	0.07
CPn0196CP0571oppA2	2	-0.29	0.12	-0.48	0.32	-0.82
CPn0195CP0572oppA1	10	-0.2	-0.9	-0.89	-0.93	-0.2
CPn0190CP0577	12	0.21	1.01	-0.42	0.62	0.81
CPn0189CP0578	12	-0.26	-1.22	-1.36	-0.62	0.22
CPn0188CP0579	11	-0.28	-1.04	-1.66	-0.48	0
CPn0180CP0588		0.03	-1.12	-1.24	0.24	0.35
CPn0176CP0593		0.12	1.18	0.76	0.69	0.61
CPn0175CP0594		-0.19	0.34	-0.16	-0.22	-0.28
CPn0170CP0600		0.43	0.92	0.72	1.06	1
CPn0169CP0602		-0.05	-0.35	-0.65	-0.46	-0.23
CPn0168CP0603	2	-0.09	1.13	1	2.23	1.39
CPn0165CP0606		-0.28	-0.47	-0.77	-0.06	-0.18
CPn0163CP0608	2	-0.01	0.94	0.18	0.88	0.33
CPn0161CP0610	10	-0.18	-0.4	-0.77	-0.56	-0.37
CPn0159CP0612		0.18	1.12	0.75	0.63	1.38
CPn0158CP0613		0.13	0.67	0.66	0.59	0.65
CPn0157CP0614		0.34	0.51	0.46	0.85	-0.14
CPn0154CP0617gseA	8	-0.62	-0.02	-0.32	-0.64	-0.44
CPn0152CP0620	2	0.16	0.23	-0.19	-0.76	-0.21
CPn0151CP0622mhpA		0	0.24	-0.37	-0.15	-0.34
CPn0150CP0623	3	0.3	0.23	0.52	0.5	0.52
CPn0149CP0624dnlJ	2	0.49	0.86	0.8	3.49	1.52
CPn0144CP0629clpB	8	0.13	-0.1	-0.08	-0.58	-0.06
CPn0143CP0630yxjG1	11	0.37	0.98	0.71	0.41	0.25
CPn0142	12	0.15	-0.6	-1.34	0.4	-0.09
CPn0140CP0632yqdE	10	0.37	-1.47	-0.53	-0.59	0.01
CPn0139CP0633yqgE	10	-0.07	-1.52	-1.34	-0.78	-0.36
CPn0138CP0634hemL	10	-0.09	-0.17	-0.63	-0.24	-0.12
CPn0137CP0635ybgI		0.29	0.6	0.23	0.93	-0.14
CPn0132CP0640	9	0.14	0.62	0.19	0.57	-0.46
CPn0131CP0641		0.22	1.52	1.14	0.97	-0.43
CPn0129CP0642	12	0.17	-0.27	-0.79	0.25	0.1
CPn0127CP0645ytfF	11	-0.12	-0.37	-0.73	-0.16	0.11
CPn0123CP0650recD1		0.18	0.83	0.44	0.31	-0.02
CPn0122CP0651metG	3	-0.04	-0.35	-0.67	-1.01	-0.55
CPn0116CP0657rs16	5	0.25	-0.53	-0.81	-1.38	-0.48
CPn0115CP0658ffh	4	-0.1	-0.21	-0.7	-1.48	-0.53
CPn0113CP0660pfrA	1	-0.1	0.69	0.44	-0.3	-0.11
CPn0112CP0661rl31	9	0.21	0.99	0.68	-0.31	0.13
CPn0108CP0666		0.65	1.17	0.19	2.04	1.04
CPn0105CP0669	10	-0.07	-0.63	-0.41	-0.44	0.15
CPn0102CP0672cydA	2	0.53	1.33	1.06	2.35	1.11
CPn0098htrB	2	0.01	0.71	-0.22	2.07	0.93
CPn0096CP0678uvrA	11	-0.17	-0.24	-0.74	-1.11	-0.37
CPn0092CP0682atpK	5	0.1	0.44	0.74	-0.09	0.1
Cpn0090_CP0684	4	0	-0.07	-0.84	-0.54	-0.86
CPn0086_CP0688	2	0.26	1.52	1.11	2.6	0.5
CPn0085CP0690	11	-0.1	-0.31	-0.17	-0.11	-0.49
CPn0084CP0691		-0.37	-0.71	-0.93	-0.38	-0.77
CPn0081CP0694rpoB	5	-0.09	-0.89	-1.3	-1.01	-0.65
CPn0072CP0703		-0.35	-0.26	-0.55	-0.2	-0.63
CPn0070		-0.26	-0.37	-0.76	0.14	-0.54
CPn0067CP0707	8	0.05	0.23	0.15	0.03	-0.7
CPn0066CP0708	2	0.33	1.89	1.49	1.94	1.72
CPn0065CP0709	12	-0.36	-1.6	-2.1	0.46	1.44
CPn0063CP0711		0.06	-0.75	-0.82	1.25	-0.04
Cpn0061_CP0714	8	-0.1	0.65	0.16	-0.19	-0.46
CPn0059CP0716dut	8	-0.33	0.14	0.06	-0.31	-0.15
CPn0055CP0720	4	0.09	0.3	-0.01	-0.69	-0.28
CPn0054CP0721rnc	2	-0.07	0.37	0.29	0.07	-0.08
CPn0053CP0722sms		0.24	0.58	0.77	0.2	0.25

NAME	Cluster in acute infection	DAM 24 h p.i.	DAM 48 h p.i.	DAM 72 h p. i.	Repleted 96 h p.i.	120 h p.i.
CPn0047	9	0	0.57	0.89	-0.17	0.44
CPn0046CP0728	2	0.28	1.13	1.04	1.66	0.74
CPn0044CP0729	8	-0.37	-0.19	0.22	0.27	-0.67
CPn0043CP0730	3	-0.12	1	0.72	2.15	0.58
CPn0041CP0733	2	-0.34	0.6	0.78	0.33	0.34
CPn0039CP0736ybaB		0	-0.24	-0.31	-0.75	-0.24
CPn0038CP0737ptsI	2	0.56	1.71	1.99	3.52	2.38
CPn0035CP0741	2	0.02	1.5	1.3	3.01	1.78
CPn0034CP0742	2	0.19	0.93	1.01	1.31	0.61
CPn0028CP0748	2	0	1.33	1.4	1.72	1.32
CPn0026CP0750	3	-0.06	0.29	0.16	1.25	0.15
CPn0025CP0751atsA	3	0.05	1.28	0.37	0.43	0.18
CPn0024CP0752xerC	2	0.28	1.3	1.01	1.58	1.42
CPn0020CP0756	2	0.28	0.94	0.67	1.64	0.32
Cpn0018_pmp51	10	-0.31	-1.05	-0.74	-1.14	-0.34
Cpn0017_pmp42	11	0.25	-0.85	-0.98	0.32	-0.14
Cpn0016_pmp41	11	-0.71	-1.4	-1.09	-0.96	-0.44
Cpn0015_pmp32	10	-0.38	-0.69	-0.67	-1.06	-0.45
Cpn0014_pmp31	10	-0.75	-1.26	-0.67	-1.56	0.09
CPn0009CP0765	2	0.15	1.21	0.85	0.93	0.69
CPn0008CP0766	2	-0.11	0.85	0.77	1.83	0.52
CPn0006	2	-0.2	1.08	1.19	2.07	1.54
CPn0004CP0771gatB	6	0.14	1.09	1.11	-0.62	-0.27
CPn0003CP0772gatA	3	0.22	0.65	0.66	0.28	-0.13
CPn0002CP0773gatC		0.18	0.52	0.84	-1.04	0.13
CPj05762CPn0576	3	-0.13	0.68	-0.01	2.94	0.2
CPj0339Cpn0339	2	0.15	0.7	0.14	2.55	0.6

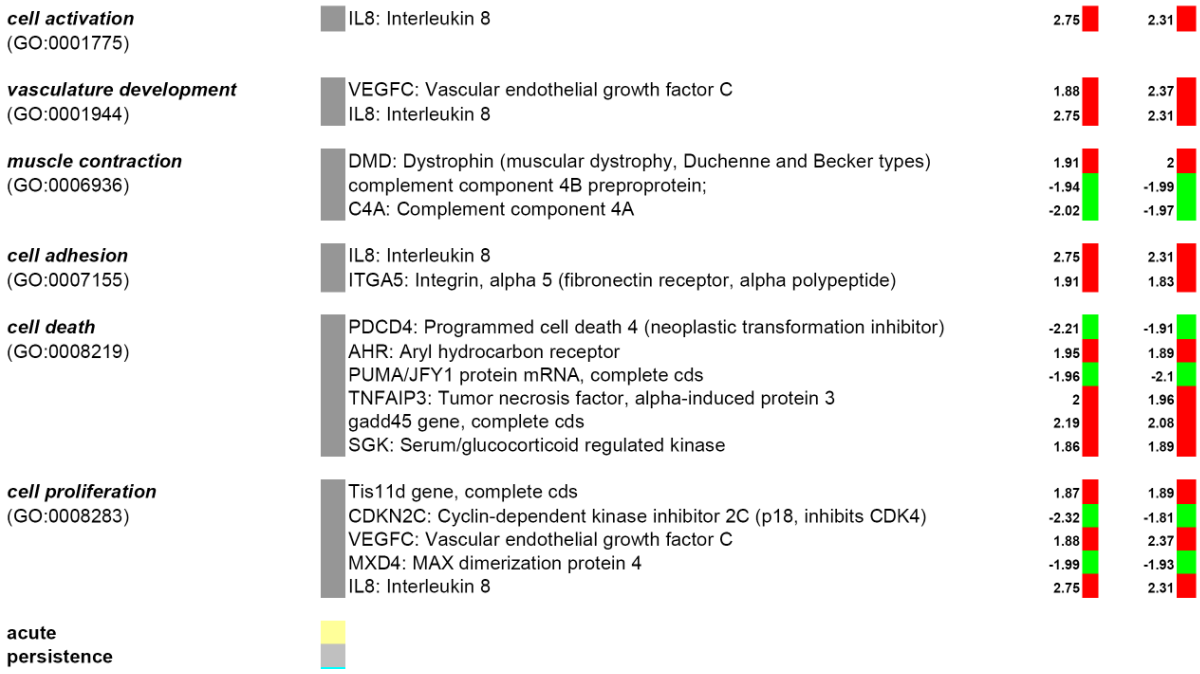
Supplementary-Table 4 - **Comparison of differentially regulated genes between the acute and iron mediated persistent infection at 4 h p.i.** The comparison has been done with FatiGO (**www.fatigo.org**) using gene ontology terms. (Note that to a certain gene, several GO terms can be connected, so that genes can show up in several groups.). Yellow indicates genes regulated in the acute and grey in the persistent infection. Red indicates up-regulation green down-regulation.

		NI vs I	UV vs I
organismal physiological process (GO:0050874)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	NR4A2: Nuclear receptor subfamily 4, group A, member 2	2.42	2.31
	LTB: Lymphotoxin beta (TNF superfamily, member 3)	-2.22	-1.89
	CXCL2: Chemokine (C-X-C motif) ligand 2	2.05	2.03
	CRX cone-rod homeobox, mRNA, complete cds	-2.11	-1.95
	PDYN: Prodynorphin	-2.63	-3.48
	FOS: V-fos FBJ murine osteosarcoma viral oncogene homolog	1.93	2.43
	SH2D1A: SH2 domain protein 1A, Duncan's disease (lymphoproliferative syndrome)	1.89	2.41
response to stimulus (GO:0050896)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	NR4A2: Nuclear receptor subfamily 4, group A, member 2	2.42	2.31
	FOS: V-fos FBJ murine osteosarcoma viral oncogene homolog	1.93	2.43
	CXCL2: Chemokine (C-X-C motif) ligand 2	2.05	2.03
	CRX cone-rod homeobox, mRNA, complete cds	-2.11	-1.95
	LTB: Lymphotoxin beta (TNF superfamily, member 3)	-2.22	-1.89
	SH2D1A: SH2 domain protein 1A, Duncan's disease (lymphoproliferative syndrome)	1.89	2.41
	CYR61: Cysteine-rich, angiogenic inducer, 61	3.85	2.00
negative regulation of biological process (GO:0048519)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	BIRC4: Baculoviral IAP repeat-containing 4	-3.11	-1.90
	ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.25	2.01
organ development (GO:0048513)	CRX cone-rod homeobox, mRNA, complete cds	-2.11	-1.95
	NRP2: Neuropilin 2	-33.56	-1.80
	FGF11: Fibroblast growth factor 11	-2.35	-2.05
cellular physiological process (GO:0050875)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	CRX cone-rod homeobox, mRNA, complete cds	-2.11	-1.95
	NR4A2: Nuclear receptor subfamily 4, group A, member 2	2.42	2.31
	NRP2: Neuropilin 2	-33.56	-1.80
	FOS: V-fos FBJ murine osteosarcoma viral oncogene homolog	1.93	2.43
	BIRC4: Baculoviral IAP repeat-containing 4	-3.11	-1.90
	TEK: TEK tyrosine kinase, endothelial	-4.18	-3.58
	CYR61: Cysteine-rich, angiogenic inducer, 61	3.85	2.00
	FZD5: frizzled homolog 5 (Drosophila) (FZD5)mRNA	-1.86	-2.20
	ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.25	2.01
	TGFBR1: Transforming growth factor, beta receptor I	-3.64	-3.21
	CYP26A1: Cytochrome P450, family 26, subfamily A, polypeptide 1	-2.65	-1.80
	OVOL1: Ovo-like 1(Drosophila)	-2.03	-1.91
	KCNJ4: Potassium inwardly-rectifying channel, subfamily J, member 4	-1.85	2.05
regulation of cellular process (GO:0050794)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	CRX cone-rod homeobox, mRNA, complete cds	-2.11	-1.95
	NR4A2: Nuclear receptor subfamily 4, group A, member 2	2.42	2.31
	FOS: V-fos FBJ murine osteosarcoma viral oncogene homolog	1.93	2.43
	BIRC4: Baculoviral IAP repeat-containing 4	-3.11	-1.90
	CYR61: Cysteine-rich, angiogenic inducer, 61	3.85	2.00
	ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.25	2.01
	OVOL1: Ovo-like 1(Drosophila)	-2.03	-1.91
regulation of physiological process (GO:0050791)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	CRX cone-rod homeobox, mRNA, complete cds	-2.11	-1.95
	NR4A2: Nuclear receptor subfamily 4, group A, member 2	2.42	2.31
	FOS: V-fos FBJ murine osteosarcoma viral oncogene homolog	1.93	2.43
	BIRC4: Baculoviral IAP repeat-containing 4	-3.11	-1.90
	CYR61: Cysteine-rich, angiogenic inducer, 61	3.85	2.00
	ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.25	2.01
	OVOL1: Ovo-like 1(Drosophila)	-2.03	-1.91
reproduction (GO:0000003)	OVOL1: Ovo-like 1(Drosophila)	-2.03	-1.91
death (GO:0016265)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	BIRC4: Baculoviral IAP repeat-containing 4	-3.11	-1.92
morphogenesis (GO:0009653)	CRX cone-rod homeobox, mRNA, complete cds	-2.11	-1.95
	NRP2: Neuropilin 2	-33.56	-1.80
	FGF11: Fibroblast growth factor 11	-2.35	-2.05
	CYR61: Cysteine-rich, angiogenic inducer, 61	3.85	2.00
metabolism (GO:0008152)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	CRX cone-rod homeobox, mRNA, complete cds	-2.11	-1.95
	NR4A2: Nuclear receptor subfamily 4, group A, member 2	2.42	2.31
	FOS: V-fos FBJ murine osteosarcoma viral oncogene homolog	1.93	2.43
	BIRC4: Baculoviral IAP repeat-containing 4	-3.11	-1.90
	TEK: TEK tyrosine kinase, endothelial	-4.18	-3.58
	OVOL1: Ovo-like 1(Drosophila)	-2.03	-1.91
	ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.25	2.01
	TGFBR1: Transforming growth factor, beta receptor I	-3.64	-3.21
	CYP26A1: Cytochrome P450, family 26, subfamily A, polypeptide 1	-2.65	-1.80
cell communication (GO:0007154)	IFNB1: Interferon, beta 1, fibroblast	-2.05	-2.31
	NR4A2: Nuclear receptor subfamily 4, group A, member 2	2.42	2.31
	LTB: Lymphotoxin beta (TNF superfamily, member 3)	-2.22	-1.89
	CXCL2: Chemokine (C-X-C motif) ligand 2	2.05	2.03
	NRP2: Neuropilin 2	-33.56	-1.80
	PDYN: Prodynorphin	-2.63	-3.48
	ADAMTS1: ADAM metallopeptidase with thrombospondin type 1 motif, 1	2.25	2.01
	TEK: TEK tyrosine kinase, endothelial	-4.18	-3.58
	CYR61: Cysteine-rich, angiogenic inducer, 61	3.85	2.00
	FGF11: Fibroblast growth factor 11	-2.35	-2.05
	TGFBR1: Transforming growth factor, beta receptor I	-3.64	-3.21
	SH2D1A: SH2 domain protein 1A, Duncan's disease	1.89	2.41
	C2orf31: Frizzled homolog 5 (Drosophila)	-1.86	-2.20

Supplementary-Table 5 - **Comparison of differentially regulated genes between the acute and iron mediated persistent infection at 24 h p.i.** The comparison has been done with FatiGO (www.fatigo.org) using gene ontology terms. (Note that to a certain gene, several GO terms can be connected, so that genes can show up in several groups). Yellow indicates genes regulated in the acute and grey in the persistent infection. Red indicates up-regulation green down-regulation.

		NI vs I	UV vs I
cellular metabolism (GO:0044237)	transcription factor TZP	?	?
	UBB: Ubiquitin B	-1.86	-1.89
	DUSP4: Dual specificity phosphatase 4	4.17302	3.3139
	ID1: Inhibitor of DNA binding 1	2.01349	1.90185
	MAP2K3: Mitogen-activated protein kinase kinase 3	1.94491	1.98135
	NR4A1: Nuclear receptor subfamily 4, group A, member 1	2.11187	1.95358
	SEPP1: Selenoprotein P, plasma, 1	-1.90473	-2.12644
	PINK1: PTEN induced putative kinase 1	-2.06966	-2.10459
	JUN: V-jun sarcoma virus 17 oncogene homolog (avian)	3.02748	2.62427
	Human l-myc gene exon 3 and 3'-flanking region	-2.15	-2.13498
	SRF: Serum response factor (c-fos serum response transcription factor)	1.85126	2.49387
	HRMT1L2: HMT1 hnRNP methyltransferase-like 2	1.86337	2.066
	ULK1: Unc-51-like kinase 1 (C. elegans)	-1.90531	-2.1515
	PTPRN2: Protein tyrosine phosphatase, receptor type, N polypeptide 2	-2.04	-2.16
	TNFAIP3: Tumor necrosis factor, alpha-induced protein 3	2	1.96
	MXD4: MAX dimerization protein 4	-1.99	-1.93
	Homo sapiens gadd45 gene, complete cds	2.19	2.08
	DDB2: LIM homeobox 3	-2.23	-1.9
	AHR: Aryl hydrocarbon receptor	1.95	1.89
	DUSP2: Dual specificity phosphatase 2	1.84	1.8
	SGK: Serum/glucocorticoid regulated kinase	1.8	1.89
	PLK2: Polo-like kinase 2	2.01	1.98
response to biotic stimulus (GO:0009607)	AOAH: Acyloxyacyl hydrolase (neutrophil)	2.57267	1.96433
	HRMT1L2: HMT1 hnRNP methyltransferase-like 2	1.86337	2.066
	SEPP1: Selenoprotein P, plasma, 1	-1.90473	-2.12644
	ANKRD1: Ankyrin repeat domain 1 (cardiac muscle)	2.90313	3.24039
	IFIT3: Interferon-induced protein with tetratricopeptide repeats 3	2.33319	2.18895
	SPINK5: Serine peptidase inhibitor, Kazal type 5	-2.56	-2.38
	IL8: Interleukin 8	2.75	2.31
	complement component 4B preproprotein	-1.94	-1.99
	C4A: Complement component 4A	-2.02	-1.97
response to external stimulus (GO:0009605)	AOAH: Acyloxyacyl hydrolase (neutrophil)	2.57267	1.96433
	THBD: Thrombomodulin	2.54859	2.20111
	PLAUR: Plasminogen activator, urokinase receptor	2.44531	2.23445
	SPINK5: Serine peptidase inhibitor, Kazal type 5	-2.56	-2.38
	IL8: Interleukin 8	2.75	2.31
	complement component 4B preproprotein;	-1.94	-1.99
	AHR: Aryl hydrocarbon receptor	1.95	1.89
	C4A: Complement component 4A	-2.02	-1.97
macromolecule metabolism (GO:0043170)	UBB: Ubiquitin B	-1.86	-1.89
	MAP2K3: Mitogen-activated protein kinase kinase 3	1.94491	1.98135
	ULK1: Unc-51-like kinase 1 (C. elegans)	-1.90531	-2.1515
	DUSP4: Dual specificity phosphatase 4	4.17302	3.3139
	PINK1: PTEN induced putative kinase 1	-2.06966	-2.10459
	HRMT1L2: HMT1 hnRNP methyltransferase-like 2	1.86337	2.066
	PTPRN2: Protein tyrosine phosphatase, receptor type, N polypeptide 2	-2.04	-2.16
	DUSP2: Dual specificity phosphatase 2	1.84	1.8
	TNFAIP3: Tumor necrosis factor, alpha-induced protein 3	2	1.96
	SGK: Serum/glucocorticoid regulated kinase	1.8	1.89
	PLK2: Polo-like kinase 2 (Drosophila)	2.01	1.98
transport (GO:0006810)	SLC1A5: Solute carrier family 1 (neutral amino acid transporter)	1.80205	2.04976
	SLC12A2: Solute carrier family (sodium/potassium/chloride transporters)	2.57863	1.96019
	RIN1: Ras and Rab interactor 1	2.07348	2.12316
	Homo sapiens aquaporin 1 (channel-forming integral protein, 28kDa)	-1.95	-1.8
	SLC19A1: Solute carrier family 19 (folate transporter), member 1	2.22	1.91
	SGK: Serum/glucocorticoid regulated kinase	1.8	1.89
	aquaporin 7	-2.2	-1.1
organogenesis (GO:0009887)	LMNA: Lamin A/C	1.93442	1.90203
	DMD: Dystrophin (muscular dystrophy, Duchenne and Becker types)	1.91	2
	IL8: Interleukin 8	2.75	2.31
	VEGFC: Vascular endothelial growth factor C	1.88	2.37
	BMP6: Bone morphogenetic protein 6	2.16	2.39

response to stress (GO:0006950)	AOAH: Acyloxyacyl hydrolase (neutrophil)	2.57267	1.96433
	PINK1: PTEN induced putative kinase 1	-2.06966	-2.10459
	SEPP1: Selenoprotein P, plasma, 1	-1.90473	-2.12644
	THBD: Thrombomodulin	2.54859	2.20111
	PLAUR: Plasminogen activator, urokinase receptor	2.44531	2.23445
	SPINK5: Serine peptidase inhibitor, Kazal type 5	-2.56	-2.38
	Homo sapiens gadd45 gene, complete cds	2.19	2.08
	AHR: Aryl hydrocarbon receptor	1.95	1.89
	complement component 4B preproprotein	-1.94	-1.99
	IL8: Interleukin 8	2.75	2.31
	DDB2: LIM homeobox 3	-2.23	-1.9
	SGK: Serum/glucocorticoid regulated kinase	1.86	1.89
	C4A: Complement component 4A	-2.02	-1.97
immune response (GO:0006955)	AOAH: Acyloxyacyl hydrolase (neutrophil)	2.57267	1.96433
	IFIT3: Interferon-induced protein with tetratricopeptide repeats 3	2.33319	2.18895
	SPINK5: Serine peptidase inhibitor, Kazal type 5	-2.56	-2.38
	IL8: Interleukin 8	2.75	2.31
	complement component 4B preproprotein;	-1.94	-1.99
	C4A: Complement component 4A	-2.02	-1.97
establishment of localization (GO:0051234)	SLC1A5: Solute carrier family 1 (neutral amino acid transporter)	1.80205	2.04976
	SLC12A2: Solute carrier f (sodium/potassium/chloride transporters)	2.57863	1.96019
	RIN1: Ras and Rab interactor 1	2.07348	2.12316
	Homo sapiens aquaporin 1	-1.95	-1.8
	SLC19A1: Solute carrier family 19 (folate transporter), member 1	2.22	1.91
	SGK: Serum/glucocorticoid regulated kinase	1.8	1.89
signal transduction (GO:0007165)	DUSP4: Dual specificity phosphatase 4	4.17302	3.3139
	MAP2K3: Mitogen-activated protein kinase kinase 3	1.94491	1.98135
	RIN1: Ras and Rab interactor 1	2.07348	2.12316
	ANKRD1: Ankyrin repeat domain 1 (cardiac muscle)	2.90313	3.24039
	LANCL1: LanC lantibiotic synthetase component C-like 1 (bacterial)	-1.89474	-1.86567
	NR4A1: Nuclear receptor subfamily 4, group A, member 1	2.11187	1.95358
	PINK1: PTEN induced putative kinase 1	-2.06966	-2.10459
	SRF: Serum response factor (c-fos serum response transcription factor)	1.85126	2.49387
	HRMT1L2: HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	1.86337	2.066
	ULK1: Unc-51-like kinase 1 (C. elegans)	-1.90531	-2.1515
	PLAUR: Plasminogen activator, urokinase receptor	2.44531	2.23445
	VEGFC: Vascular endothelial growth factor C	1.88	2.37
	RGS2: Regulator of G-protein signalling 2, 24kDa	2.2	2.08
	GNG11: Guanine nucleotide binding protein (G protein), gamma 11	2.3	1.87
	IL8: Interleukin 8	2.75	2.31
	AHR: Aryl hydrocarbon receptor	1.95	1.89
	DUSP2: Dual specificity phosphatase 2	1.84	1.8
	ITGA5: Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	1.9	1.83
	PLK2: Polo-like kinase 2 (Drosophila)	2.01	1.98
cell cycle (GO:0007049)	DUSP4: Dual specificity phosphatase 4	4.17302	3.3139
	CCNG2: Cyclin G2	-1.89773	-2.32207
	VEGFC: Vascular endothelial growth factor C	1.88	2.37
	IL8: Interleukin 8	2.75	2.31
	CDKN2C: Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	-2.32	-1.81
	Homo sapiens gadd45 gene, complete cds	2.19	2.08
	RGS2: Regulator of G-protein signalling 2, 24kDa	2.2	2.08
	AHR: Aryl hydrocarbon receptor	1.95	1.89
	PLK2: Polo-like kinase 2 (Drosophila)	2.01	1.98
blood coagulation (GO:0007596)	THBD: Thrombomodulin	2.54859	2.20111
	PLAUR: Plasminogen activator, urokinase receptor	2.44531	2.23445
cell organization and biogenesis (GO:0016043)	KRT7: Keratin 7	2.56951	2.51522
regulation of body fluids (GO:0050878)	THBD: Thrombomodulin	2.54859	2.20111
	PLAUR: Plasminogen activator, urokinase receptor	2.44531	2.23445
cell division	CCNG2: Cyclin G2	-1.89773	-2.32207


cell activation
(GO:0001775)
IL8: Interleukin 8
2.75
2.31
vasculature development
(GO:0001944)
VEGFC: Vascular endothelial growth factor C
1.88
2.37
IL8: Interleukin 8
2.75
2.31
muscle contraction
(GO:0006936)
DMD: Dystrophin (muscular dystrophy, Duchenne and Becker types)
1.91
2
complement component 4B preproprotein;
-1.94
-1.99
C4A: Complement component 4A
-2.02
-1.97
cell adhesion
(GO:0007155)
IL8: Interleukin 8
2.75
2.31
ITGA5: Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
1.91
1.83
cell death
(GO:0008219)
PDCD4: Programmed cell death 4 (neoplastic transformation inhibitor)
-2.21
-1.91
AHR: Aryl hydrocarbon receptor
1.95
1.89
PUMA/JFY1 protein mRNA, complete cds
-1.96
-2.1
TNFAIP3: Tumor necrosis factor, alpha-induced protein 3
2
1.96
gadd45 gene, complete cds
2.19
2.08
SGK: Serum/glucocorticoid regulated kinase
1.86
1.89
cell proliferation
(GO:0008283)
Tis11d gene, complete cds
1.87
1.89
CDKN2C: Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
-2.32
-1.81
VEGFC: Vascular endothelial growth factor C
1.88
2.37
MXD4: MAX dimerization protein 4
-1.99
-1.93
IL8: Interleukin 8
2.75
2.31
acute
persistence