Transcription in *Mycoplasma pneumoniae*

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Für meine Eltern,

Else Eilers und Hermann Eilers (†)

I hereby declare that the doctoral thesis entitled, “Transcription in *Mycoplasma pneumoniae*” has been written independently and with no other sources and aids than quoted.

Hinnerk Eilers

1. Introduction

„It is not the strongest of the species that survives, nor the most intelligent, but rather the one most adaptable to change.“

Clarrence Darrow, 1988

Bacteria, unicellular and rather simple organisms, are the most abundant life form on earth. It is estimated that $4 – 6 \times 10^{30}$ individual bacterial cells can be found on earth, representing the largest pool of nitrogen and phosphate and the second largest pool of carbon stored in all organisms (Whitman *et al.*, 1998). Bacteria can be found ubiquitously in all possible environments. During evolution, they have developed a large metabolic diversity to adapt to even extreme conditions, often making them the dominant species in these ecological niches. Even though being highly adapted to their favoured habitats, all bacteria have to face changes in their environment and rely on sensory and regulatory mechanisms to cope with these changes. Studying these mechanisms on the molecular level is of growing importance. It allows understanding how pathogenic bacteria adapt to their hosts, to develop new drugs, molecular tools for research and to control biosynthesis in biotechnical processes. Most important, recent findings suggest that many regulatory mechanisms are conserved in all three domains of life. Therefore studying regulatory mechanisms in bacteria provides an insight into possible mechanisms in higher organisms, yet in systems of lower complexity.
This work focuses on sensory and regulatory mechanisms in one of the simplest organisms known, the human pathogen *Mycoplasma pneumoniae*.

1.1 Signal transduction in bacteria

Bacteria have developed a broad variety of mechanisms to react to changes in their environment. All mechanisms have in common that an external stimulus is sensed and transduced into a cellular response, either adjustments of enzymatic or physiological activity or alteration of gene expression, aiming to adapt to these new conditions. The stimuli sensed, as well as the output responses, can both be specific or general. As an example, the zinc uptake repressor Zur in the soil bacterium *Bacillus subtilis* specifically senses intracellular Zn^{2+} concentrations and mediates specific responses towards zinc starvation, whereas the expression and activity of general stress-response sigma factor SigS in *Escherichia coli* is triggered by various signals (Gaballa and Helmann, 1998; Hengge-Aronis, 2002). Cellular

responses can appear in all stages of bacterial gene expression (see Fig. 1.1 for an overview of most common mechanisms of regulation). Furthermore, sensing and transduction systems are often coupled to allow fast responses. Since discussing bacterial sensing systems would go beyond the scope of this work, only the most common regulatory mechanisms in bacteria are described in the next chapters.

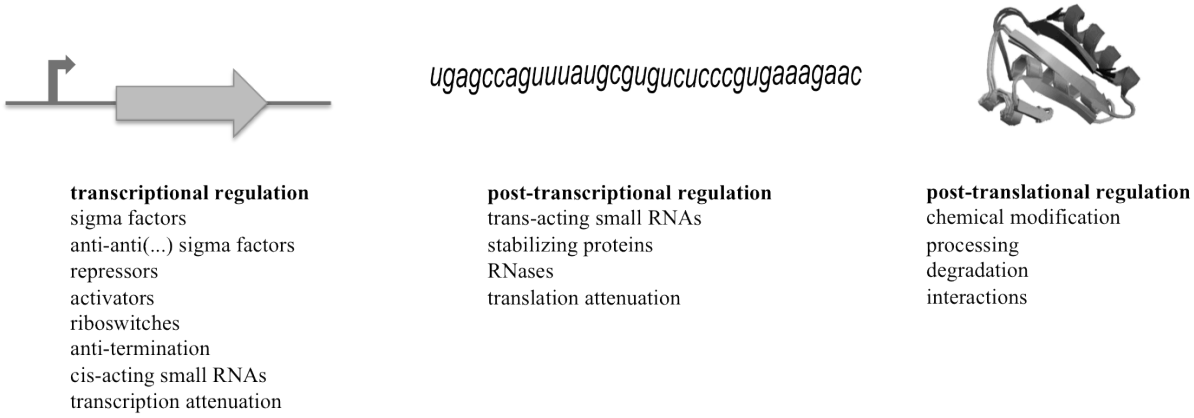


Fig. 1.1: Regulatory mechanism in bacteria

Examples of the most common mechanisms how bacteria regulate gene expression are given in the figure above.

1.1.1 Transcription regulation in bacteria

Regulation of transcription is the most prevalent mechanism to respond to environmental changes in bacteria. It allows adjusting gene expression without unnecessarily wasting energy and other resources. Transcription can be regulated by several different mechanisms and often genes are targets of more then one regulatory event.

In most bacteria the largest regulons, i.e. groups of genes that are under control of the same regulator, are those that are transcribed under the control of specific sigma factors. Sigma factors are proteins that initiate transcription by recruiting RNA polymerase to promoters of genes. They consist of one, in exceptional cases two proteins (Gruber and Gross, 2003; Paget and Helmann, 2003; MacLellan *et al.*, 2009) with two conserved helix-turn-helix motifs that recognize and bind to specific promoter sequences (Gruber and Gross, 2003). Often sigma factors themselves are targets of regulatory events, either on the level of expression like *B. subtilis* SigD (Mirel *et al.*, 2000), or by inactivation through interactions with so called anti-sigma factors. Such anti-sigma factors can either be proteins like FlgM in the case of *B. subtilis* SigD, or other molecules like 6S RNA in *E. coli* (Fredrick and Helmann, 1996; Trotochaud and Wassarman, 2004). Besides sigma and anti-sigma factors, other proteins or molecules can also trigger the affinity of RNA polymerase towards specific promoters.

Examples for this are the transcription regulator Spx present in the *Firmicutes,* or the alarmone (p)ppGpp (both discussed later) that interact with the RNA polymerase and, by this, mediate transcription regulation.

Transcription regulators are responsible for the majority of transcription regulatory events. These regulators are proteins with DNA binding domains such as helix-turn-helix motifs (Brennan and Matthews, 1989) or zinc-finger domains that bind near the regulated promoters. Transcription regulators can be divided into three groups: activators, repressors and pleiotropic regulators that can act both as repressor or activator. Activators can act by several different mechanisms. However, the majority of these proteins bind to operator sequences upstream of their target promoters. Binding to their operator either results in changes of the topology of the DNA, allowing RNA polymerase to bind to the promoter, or to additional, *cis*-acting elements. Other activators are affecting the half-lives of open promoter complexes, thereby abetting transcription initiation. Although transcription activators that bind downstream of the +1 site of transcription have been found (Sperandio *et al.*, 2000; Munson and Scott, 2000), they are more likely an exception from the classical activating mechanisms of this group of proteins. In contrast to activators, repressors are DNA binding proteins that bind directly to the promoter or in the leader region between the promoter and the start codon of their target gene. When binding to the promoter region, repressors either cover the +1 site, the -10 or -35 regions, thereby preventing sigma binding or melting of DNA to start transcription. Repressors that bind to the leader sequence of their target gene block the active RNA polymerase, thereby leading to a break-off of transcription, a mechanism refered to as ´roadblock´ (Kim *et al.*, 2002; Choi and Saier, 2005). Finally, pleiotropic regulators such as the carbon catabolite control protein CcpA in the *Firmicutes* are DNA binding proteins that act both as activators or repressors, depending on their binding site in respect of the controlled gene (Blencke *et al.*, 2003). Moreover, DNA binding of several competing regulators to a common operator can affect their activity by antagonizing effects, as shown for the *lev* operon in *B. subtilis* (Martin-Verstraete *et al.*, 1995). Apart from these specialized proteins whose only function is to sense environmental changes and to respond by governing transcription, other proteins like *B. subtilis* RocG can moonlight as transcription regulators (Commichau *et al.*, 2007). Based on their bi-functional role as enzymes and transcription regulators, these proteins are referred to as ´trigger enzymes´.

RNA switches are *cis*-acting regulatory RNA elements that control transcription or translation by anti-termination. They are located in the 5´leader sequence of transcripts and form a terminator structure in the absence of their effector, leading to transcription breakup. If the

effector is present, it binds to the transcription terminator leading to structural changes and continuing transcription. Several molecules have been described as riboswitch effectors, including tRNA, proteins, and metabolites. In addition, riboswitches that do not require effector binding but other stimuli, like the temperature sensitive riboswitch upstream of *Listeria monocytogenes prfA*, have been discovered (Johansson *et al.*, 2002). Recent studies found that at least 1.6% of all genes in *B. subtilis* and 1.4% of all genes in *L. monocytogenes* are controlled by riboswitches, highlighting the importance of these elements in bacterial transcription regulation (Irnov *et al.*, 2006; Toledo-Arana *et al.*, 2009).

1.1.2 Post-transcriptional regulation in bacteria

For a long period, post-transcriptional regulations where thought to occur only by RNA processing or degradation. However, during the last years, another widely distributed mechanism of post-transcriptional control has been discovered involving small, non-coding RNAs (sRNA). These sRNAs can be grouped in two categories, according to their mechanism of regulation: (I) small, trans-acting antisense sRNAs that bind to their target mRNA by base pairing and (II) sRNAs that interact with proteins, thereby triggering their activity. An example of group (II) is *E. coli* 6S RNA, described previously. sRNAs of group (I) can either effect translation initiation by masking or exposing the Shine-Dalgarno sequence of its target mRNAs (e.g. *B. subtilis* SR1) or by altering the stability of their targets as for *Staphylococcus aureus* RNAIII (Heidrich *et al.*, 2006; Huntzinger *et al.*, 2005). Moreover, recent publications showed that also small mRNAs could function as regulatory RNAs, and that titration effects trigger regulatory effects of sRNA with multiple targets (Mangold *et al.*, 2004; Gimpel *et al.,* 2010; Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009). Full operational capability of sRNAs in Gram-negative bacteria requires the activity of the RNA chaperone Hfq that stabilizes these sRNAs or mediates duplex formation of sRNA and its target mRNA (Soper *et al.*, 2010). Even though an Hfq ortholog is present in most Gram-positive bacteria, it is not required for stabilisation of sRNA and its function is so far unknown (Heidrich *et al.*, 2006).

Gene expression can also be regulated by transcript stability. As described previously, stability of RNA can be affected by interactions with other RNA molecules or proteins. In addition to this, some RNAs are targets of specific ribonucleases. For example, the *gapA* operon of *B. subtilis* is transcribed as a polycistronic RNA of six genes and post-transcriptionally processed by endoribonuclease RNaseY, leading to the formation of two mature mRNA fragments with different stability (Ludwig *et al.*, 2001; Commichau *et al.*,

2009). The stability of the shorter, *gapA* harbouring mRNA is triggered by a small peptide coded on sRNA S1 by an unknown mechanism (Gimpel *et al.*, 2010).

3´polyadenylation of transcripts is a third mechanism to regulate transcript stability in bacteria. In contrast to eukaryotes, 3´polyadenylation was found to lead to quicker RNA degradation in bacteria instead of stabilizing transcripts (Marujo *et al.*, 2000). In *E. coli*, transcripts of over 90% of open reading frames where shown to be polyadenylated during exponential growth, and recent publications describe a poly(A) dependent mechanism of *glmS* regulation in this bacterium, suggesting a common role of polyadenylation in regulating gene expression in *E. coli* (Mohanty and Kushner, 2006; Joanny *et al.*, 2007). Whereas poly(A) polymerase I (PAP I) and, to a lesser extend, polynucleotide phosphorylase (PNPase) have been found to be responsible for polyadenylation of transcripts in *E. coli*, synthesis of poly(A) tails in *B. subtilis* depends on the activity of a so far unknown enzyme (O´Hara *et al.*, 1995; Campos-Guillén *et al.*, 2005). Moreover, the role of polyadenylation in RNA stability and regulation in Gram-positive bacteria has not been studied so far.

1.1.3 Post-translational regulation in bacteria

Post-translational regulations in bacteria occur mainly by three mechanisms: protein modification, protein degradation or by interaction with proteins or other molecules. Protein modifications can either take place by protein processing, rather rarely in bacteria, or by the addition of chemical groups to certain amino acid residues of the protein. A lot of different chemical post-translational protein modifications have been described in bacteria, including phosphorylation, acetylation, methylation, uridylylation/adenylation and glycolysation (Cozzone, 1998; Wang *et al.*, 2010; Amaro and Jerez**,** 1984; Edwards and Merrick, 1995; Abu-Qarn *et al.*, 2008). These chemical modifications are either directly involved in regulatory events or in signal transduction by triggering enzymatic activity (Wang *et al.*, 2010), mediating interactions with proteins or other molecules such as RNA (Deutscher *et al.*, 1995; Lindner *et al.*, 1999) or affecting the stability of their target proteins (Schmidl *et al.*, 2010). Post-translational regulations in a quantitative manner can also occur by targeted degradation of proteins. For example, the Clp protease complex in *B. subtilis* was shown to preferably degrade proteins involved in central metabolism in response to glucose starvation, thereby down regulating these pathways (Gerth *et al.*, 2008). This protease complex is also responsible for the instability of transcription regulator Spx in this bacterium, as will be discussed later.

1.2 The *Mollicutes* and the organism *Mycoplasma pneumoniae*

Although a multitude of distinct bacteria have been studied to date, the common model organisms for Gram-positive and Gram-negative bacteria are the soil bacterium *B. subtilis* and the enterobacterium *E. coli*, respectively. Even though being studied for more than seventy years, only recent publications in the field of synthetic biology have attracted wide attention to the *Mollicutes* and their subgroup, the *Mycoplasmas*. In the next two chapters, the *Mollicutes* in general and the organism *M. pneumoniae* in particular will be described.

1.2.1 The class *Mollicutes*

The class *Mollicutes* forms a subgroup within the phylum of the *Firmicutes*, Gram-positive bacteria with a low GC content (Ciccarelli *et al.*, 2006). The name *Mollicutes* is derived from the Latin words *mollis*, meaning "soft" and *cutis*, meaning "skin", in reference to the fact that members of this subgroup lack a cell wall (Gibbons and Murray, 1987). To date, this group consists of the genera *Mycoplasma*, *Acholeplasma*, *Spiroplasma*, *Mesoplasma*, *Hemoplasma*, *Phytoplasma* and *Ureaplasma*. 16S rRNA analysis has shown that the *Mollicutes* arose by degenerative evolution from a clostridial ancestor, common to the branches *Bacillus* and *Lactobacillus* (Woese *et al.*, 1979). Phylogenetic studies on the amino acid sequences of the widely distributed enzyme phosphoglycerate kinase (Pgk) within these groups could also reveal a closer relationship of the *Mollicutes* to the *Streptococcus/Lactobacillus* branch of *Firmicutes*, suggesting a shared ancestor rather with this subgroup than with the groups *Bacillus* and *Clostridium* (Neimark, 1979; Wolf *et al.*, 2004).

As a result of their reductive evolution, all *Mollicutes* possess highly reduced genomes, ranging from 1.5 Mbp in *Acholeplasma laidlawii* to 0.58 Mbp in *Mycoplasma genitalium*, the bacterium containing the smallest genome able to grow independent of a host (NCBI; Fraser *et al.*, 1995). These reduced genomes are also reflected by their lifestyle: all members of this group are adapted to live as parasites in close association with eukaryotic cells (Ochman and Davalos, 2006) and to date, some members like the *Phytoplasma* can still not be grown *in vitro*. Their small genomes make the *Mollicutes*, especially the *Mycoplasmas* an important model organism of the comparatively young sciences of synthetic biology and systems biology. *M. genitalium* was used to study the minimal genetic requirements of an independent living cell, and recent publications aimed, for the first time, to completely understand a minimal organism by intensively studying the transcriptome, metabolome and interactome of *M. pneumoniae* (Hutchison *et al.*, 1999; Glass *et al.*, 2006; Güell *et al.*, 2009; Kühner *et al.*,

2009; Yus *et al.*, 2009; Suthers *et al.* 2009). Furthermore, *M. genitalium*, *Mycoplasma mycoides* LC and *Mycoplasma capricolum* where used as model organisms to develop methods to synthesize, assemble and transplant whole bacterial genomes, important steps on the way to create artificially designed microorganisms (Lartigue *et al.*, 2007; Gibson *et al.*, 2008; Lartigue *et al.*, 2009; Bender *et al.*, 2010; Gibson *et al.*, 2010).

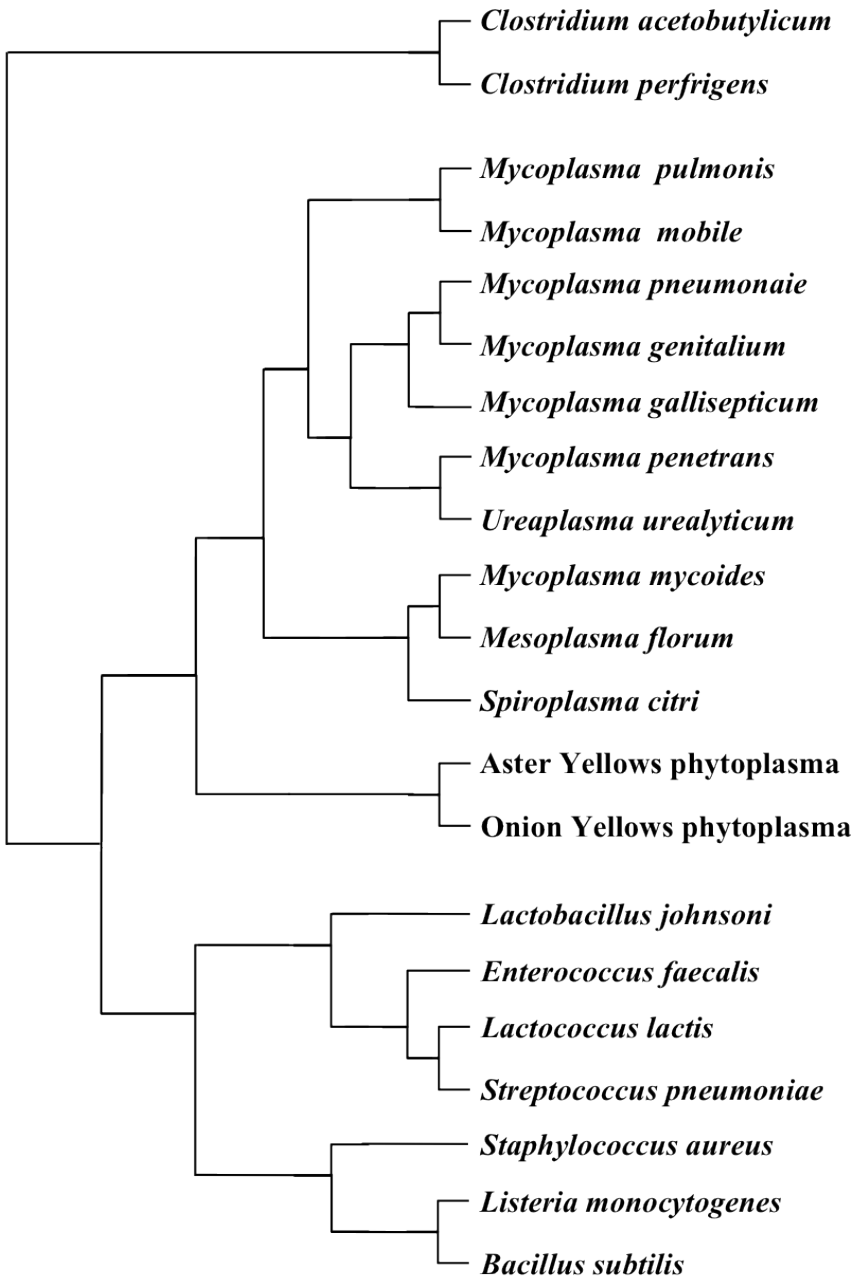


Fig. 1.2: Phylogeny of the *Mollicutes*

Unrooted phylogenetic tree of the *Firmicutes* with special emphasis to the *Mollicutes*. The tree is based on a concatenated alignment of 31 universal protein families. Image taken from Stülke *et al.*, 2009.

1.2.2 Virulence of *Mollicutes*

As mentioned above, all members of the *Mollicutes* are parasites of eukaryotic hosts. Apart from harming their hosts by cadging their nutrients, a small set of other mechanisms that lead to pathogenicity has been described. Hydrogen peroxide is the primary virulence factor in *M. pulmonis*, *M. mycoides* and *M. pneumoniae* (Cohen and Somerson, 1967; Brennan and Feinstein, 1968; Cherry and Taylor-Robinson, 1970). In *M. pneumoniae* and *M. mycoides*, hydrogen peroxide originates as a by-product from glycerol metabolism, where glycerol-3-phosphate is converted to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase, GlpD (Pilo *et al.*, 2005; Hames *et al.*, 2009). Recent studies showed that physical contact between these two *Mycoplasma* species and their host cells is required to establish cytotoxicity, suggesting a model in which hydrogen peroxide is directly injected into the host cells rather than just being secreted (Bischof *et al.*, 2008; Schmidl *et al.*, 2010). In addition to hydrogen peroxide, putative cytotoxins have been described in *M. pneumoniae* and *M. penetrans*. The *M. pneumoniae* CARDS toxin resembles the major cytotoxin of *Bordatella pertussis* in domains and enzymatic activity and was shown to cause pertussis-like symptoms in baboon and mouse infection models (Kannan and Baseman, 2006; Hardy *et al.*, 2009). The related toxin of *M. penetrans* is an ADP-ribosylating enzyme, but its role in virulence has not been determined so far (Johnson *et al.*, 2009). Despite these two toxins, the lack of obvious virulence factors in the *Mollicutes* reflects their evolution as parasites rather than opportunistic pathogens: To date, *Mycoplasma alligatoris* was reported to be the only member of the *Mollicutes* being lethal for its host, the american alligator (Brown *et al.*, 2001). Some mechanisms to escape their host's immune responses are found in the *Mollicutes*. Antigen variation is described for lipoproteins and adhesins of *M. pneumoniae*, *M. pulmonis* and several other *Mycoplasmas* (Watson *et al.*, 1988, Kenri *et al.*, 1999). In addition, several *Mycoplasma* species are able to invade and to grow within non-phagocytic cells *in vitro* (Taylor-Robinson *et al.*, 1991; Jensen *et al.*, 1994, Winner *et al.*, 2000, Yavlovich *et al.*, 2004). This behaviour could help these bacteria to hide from their host´s defence systems. However, it is not clear if intracellular growth does also occur during infection *in vivo*.

Transmission of bacterial cells to a new host occurs either by direct contact of infected with uninfected hosts or by aerosols. In addition, several *Mollicutes* have developed specific strategies to infect new hosts. *M. genitalium* is able to attach to human spermatozoa and *M. hominis* invades *Trichimonas vaginalis*, both using these mobile eukaryotic cells as a shuttle between two hosts (Svenstrup *et al.*, 2003; Vancini *et al.*, 2008). Other *Mollicutes*, such as

Phytoplasma or phytopathogenic *Spiroplasma* depend on insect shuttle vectors for transmission (Bové *et al.*, 2003).

1.2.3 The organism *M. pneumoniae*

M. pneumoniae is a human pathogen and the causative organism of atypical pneumonia and tracheobronchitis in children and old or immune-suppressed adults (Chanock *et al.*, 1963). It was first isolated in1944 from the sputum from a patient suffering on pneumonia-like symptons, at the time misinterpreted as a virus and only later characterized as a pleuro-pneumonia-like organism (PPLO) (Meiklejohn *et al.*, 1944; Chanock *et al.*, 1962). Almost 20 years after its first description, the name *M. pneumoniae* was proposed (Chanock, 1963).

M. pneumoniae has a bipolar cell shape consisting of the so called tip structure or attachment organelle, the major cell body and the trailing filament on the opposite site of the tip structure (see Fig.1.3). The tip structure consists of a multitude of hydrophobic adhesines such as P40 or HMW (high molecular weight) proteins that are assembled to form an electron-dense core, visible by thin-section electron microscopy (Seto and Miyata, 2003). It is required for attachment and motility of the cells and it was shown that activity of protein kinase C is required for assembly or stabilisation of involved proteins (Krause and Balish, 2001; Henderson and Jensen, 2006; Schmidl *et al.*, 2010). The trailing filament on the opposite pole of the cell is variable in length and is a remainder of cell division.

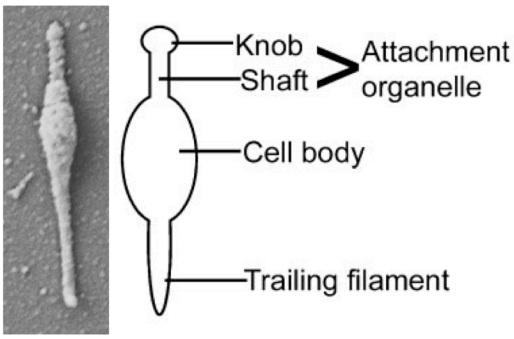


Fig. 1.3 Morphology of *M. pneumoniae*.

Scanning electron microscopy picture of *M. pneumoniae* strain M129 used in this study.

The genome of *M. pneumoniae* was a GC content of 40% and a coding capacity of only 689 putative open reading frames (Himmelreich *et al.*, 1996; Dandekar *et al.*, 2000). Furthermore, its genome sequence revealed an extremely reduced metabolic potential: *M. pneumoniae* lacks genes required for the *de novo* synthesis of amino acids and vitamins. Other metabolic

pathways, such as the pentose phosphate pathway or anabolic pathways for nucleotide synthesis are incomplete, leading to various auxotrophies and high nutrient demands such as for purines and cholesterol, when grown *in vitro*. (Johnson and Somerson, 1980; Yus *et al.*, 2009). Enzymes of the tricarboxylic acid cycle, chinones and cytochromes are missing, and energy in form of ATP can only be synthesized by substrate level phosphorylation during glycolysis. The ATPase present in the genome of *M. pneumoniae* and other *Mycoplasma* was proposed to function as an ATP-dependent proton pump rather than as an ATP generating enzyme (Linker and Wilson, 1985). Even though *M. pneumoniae* possesses uptake systems for glucose, glycerol, fructose, glycerol-3-phosphate, mannitol, ribose/galactose and ascorbate, only the first three named carbon sources promote growth *in vitro* (Halbedel *et al.*, 2004; Yus *et al.*, 2009).

In contrast to other members of the *Mollicutes*, quite a few tools to manipulate and study *M. pneumoniae* are available. Reporter systems such as *lacZ* or green fluorescent protein (GFP) fusions have been used to monitor gene expression, to analyse promoters or to locate proteins (Halbedel and Stülke, 2006; Halbedel *et al.*, 2007; Hasselbring and Krause, 2007). Targeted gene knockout by homologous recombination was shown to be working in *M. pneumoniae*, though with very low efficiency (Sluijter *et al.*, 2009; Krishnakumar *et al.*, 2010). To date, the most common method for targeted gene knockout is a method referred to as the Haystack mutagenesis, in which desired clones are detected and isolated from a pool of random transposon insertion mutants by using a PCR-based screening system (Halbedel *et al.*, 2006). Neither naturally occurring nor constructed plasmids have been described for *M. pneumoniae* or other members of the *M. pneumoniae* clade of the *Mollicutes*, even though there are plasmids available for other *Mollicutes* such as *Mycoplasma pulmonis* and *Spiroplasma citri* (Cordova *et al.*, 2002; Lartigue *et al.*, 2002). Several studies use *in vitro* assays to characterize *M. pneumoniae* enzymes and proteins. The proteins are either derived by directly expressing tagged proteins in *M. pneumoniae* or from heterologous hosts like *E. coli* (Schmidl *et al.*, 2007). For the direct expression of genes in *M. pneumoniae*, either constitutively active or TetR/tetracycline regulated promoters can be used (Schmidl *et al.*, 2007; Kühner *et al.*, 2009; Breton *et al.*, 2010). Expression of *M. pneumoniae* genes in other bacteria requires mutation of the TGA codon since it is coding for tryptophan instead of acting as a translational stop codon in this and other *Mollicutes* (Schaper *et al.*, 1987).

1.2.4 Transcription in *M. pneumoniae*

Transcription in *M. pneumoniae* basically resembles the principles in other bacteria in terms of mechanisms. However, some differences have been observed, either due to its reduced genome or to mutations in the transcription apparatus. All components of the RNA polymerase known in other bacteria are present in the genome of *M. pneumoniae* (Himmelreich *et al.*, 1996; Dandekar *et al.*, 2000). In addition, the amino acid sequences of some of the RNA polymerase subunits show mutations in conserved regions, leading to important phenotypes such as rifampine tolerance (Gadeau *et al.*, 1986). Transcription is initiated by only one sigma factor, the housekeeping sigma70 SigA. Promoters recognized by this sigma factor have a weakly conserved consensus sequence for the -10 region, consisting of TANNAT (with N=T/A/G). The -35 region is only weakly conserved in a subset of promoters and plays no significant role in transcription initiation *in vivo* (Weiner 3rd *et al.*, 2000; Halbedel *et al.*, 2007; Güell *et al.*, 2009). Promoters are mainly located in short distances to their corresponding genes, leading to short leader sequences, and often multiple transcription start points can be found upstream of genes or operons, resulting in mRNA length polymorphism (Weiner 3rd *et al.*, 2000; Halbedel *et al.*, 2007; Güell *et al.*, 2009). The majority of transcripts are polycistronic due to the high coding density of the genome and the orientation of the genes, and formation of new operons was found for several transcripts under changing environmental conditions. In addition, a high frequency of antisense transcription is observed in *M. pneumoniae* and its close relative, *M. genitalium*, probably due to unspecific promoter-like sequences that can be recognized by the sigma factor (Lluch-Senar *et al.*, 2007; Güell *et al.*, 2009).

The transcription terminator protein Rho described in other bacteria is missing in the genome of *M. pneumoniae*. This leads to transcription termination by hairpin formation as the only possible known mechanism in this organism (Himmelreich *et al.*, 1996; Weiner 3rd *et al.*, 2000; Epshtein *et al.*, 2010). *In silico* studies about Rho-independent transcription terminators in *M. pneumoniae* and other *Mollicutes* show contrary results, either doubting or supporting the presence and functionality of transcription termination by hairpin formation (de Hoon *et al.*, 2005; Washio *et al.*, 1998). However, recent publications show that the majority of transcripts in *M. pneumoniae* indeed terminate at predicted hairpins *in vivo* (Güell *et al.*, 2009; Vivancos *et al.*, 2010). A low Gibbs´ free energy density of stem loop formation in all *Mollicutes* might be the reason why the majority of Rho-independent transcription terminators have been missed in previous studies (de Hoon *et al.*, 2005).

In summary, transcription in *M. pneumoniae* seems to proceed in a relaxed mode and leads to a model in which transcription can initiate and stop at various regions in the genome.

1.2.5 Putative transcription regulators in *M. pneumoniae*

This work only focuses on genes that might directly be involved in transcription regulation. Therefore RNases or RNA binding proteins that can alter transcript stability are not further considered neither in this chapter nor in this work.

A model of relaxed transcription as described above requires tight regulation of genes that might be toxic for the cell or whose uncontrolled expression interferes with fundamental cellular processes, such as cell division. Interestingly, only very few genes that might be involved in the regulation of gene expression are found in *M. pneumoniae* (Himmelreich *et al.*, 1996):

Mpn124/HrcA

Open reading frame *mpn124* encodes an orthologue of the heat shock repressor protein HrcA in Gram-positive bacteria. HrcA is best described in *B. subtilis*, where it represses the transcription of class 1 heat-shock genes at low or moderate temperatures by binding to the so called CIRCE (controlling inverted repeat of chaperone expression) element at the corresponding promoters (Zuber and Schumann, 1994; Schulz and Schumann, 1996). At elevated temperatures, the HrcA homodimer dissociates into its subunits, thereby loosing DNA binding activity and leading to enhanced transcription of regulated genes. The 9bp-N_9-9bp CIRCE element is present at promoters of chaperones and proteases belonging to class 1 heat-shock proteins in *M. pneumoniae* and other *Mycoplasmas*, and HrcA binds to this elements *in vitro* (Chang *et al.*, 2008). In addition, up-regulation of transcription of these genes was observed after exposing *M. pneumoniae* and its relative *M. genitalium* to elevated temperatures, suggesting the same mode of regulation as described for *B. subtilis* (Weiner 3rd *et al.*, 2003; Musatovova, *et al.*, 2006). Therefore HrcA is the only transcription regulator among all *Mollicutes* whose putative targets are known so far.

Mpn239/GntR family

Open reading frame *mpn239* encodes a putative helix-turn-helix transcriptional regulator belonging to the GntR family. This family is named after the repressor of the gluconate operon in *B. subtilis* (Fujita and Fujita, 1987). To date, more than 8.500 different protein

sequences from all bacterial clades are documented in the *Pfam* database (http://pfam.sanger.ac.uk), making it the largest family of transcription regulators in bacteria. The GntR family divides into six sub-groups, namely AdR, HutC, PlmA, MocR, YtrA and AraR, and all members described so far are metabolite responsive proteins involved in the regulation of various metabolic pathways or virulence (Rigali *et al*., 2002; Hoskisson and Rigali, 2009). BLAST analysis and multiple alignments show that Mpn239 belongs to the MocR sub-family, regulators that are thought to bind to DNA in an unusual head-to-tail conformation (Rigali *et al.*, 2002; this work). The targets of this transcription regulator are so far unknown.

Mpn329/Fur family

Open reading frame *mpn329* encodes a protein of the Fur (ferric uptake repressor) family present in Gram-positive bacteria. This family is named after *B. subtilis* Fur, a Fe^{2+} responsive transcriptional repressor, that regulates the expression of siderophores and iron uptake systems in response to iron depletion. Members of this family require Fe^{2+} as a cofactor for homodimerization and thus to obtain DNA binding activity. The active homodimers bind to an 19bp operator harbouring two overlapping, similar 7bp-N-7bp inverted repeats, thereby repressing transcription of their corresponding genes (Baichoo and Helmann, 2002; Jacquamet *et al.*, 2009). Three members of the Fur family in Gram-positive bacteria have been described: Fur, Zur (zinc uptake repressor) and PerR (peroxide stress response regulator).

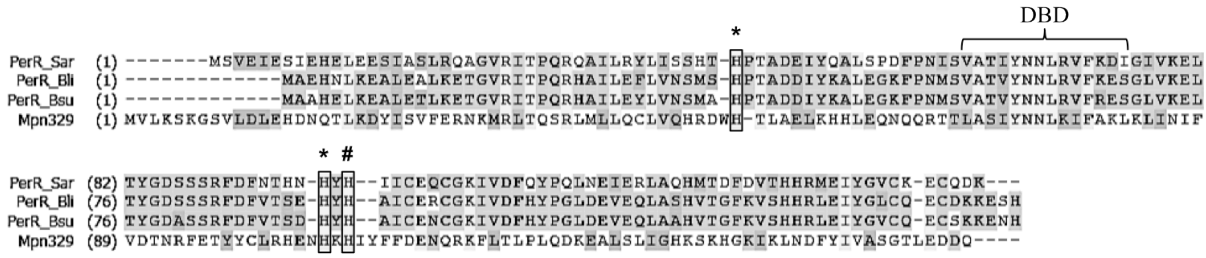


Fig. 1.4: Mpn329/Fur is related to PerR in the *Firmicutes*

Multiple alignment of PerR proteins from *S. aureus* (Sar), *B. lichiniformis* (Bli) and *B. subtilis* (Bsu) with Mpn329. The DNA binding domain (DBD) required for recognition of the PerR box and histidines His37 and His91 (*) required for H_2O_2 sensing are conserved in the *M. pneumoniae* protein. In addition, His93 (#) required for Fe^{2+} binding is conserved (Jacquamet *et al.*, 2009).

Multiple alignments with the amino acid sequences of Mpn329 and Fur, Zur or PerR proteins from other *Firmicutes* show, that the DNA binding domain and conserved histidine residues of PerR proteins are conserved in the *M. pneumoniae* protein, suggesting a function as a

peroxide response regulator rather than in iron homeostasis (Fig. 1.4) (Lee and Helmann, 2006; Jacquamet *et al.*, 2009). Even though the DNA binding domain of Mpn329 resembles those of PerR in the *Firmicutes*, no putative PerR box is found in the genome of *M. pneumoniae*. The target genes of this regulator are so far unkown.

Mpn266/Spx family

Open reading frame *mpn266* encodes an orthologue of the pleiotropic transcriptional regulator Spx of disulphide stress in Gram-positive bacteria. Its presence in the genome of *M. pneumoniae* has been missed until recently, due to the fact that it was misannotated as an ArsC (arsenate reductase) family member described in *E. coli* (Kühner *et al.*, 2009; Güell *et al.*, 2009; this work). In *B. subtilis* in which Spx is described best, Spx interacts with the C-terminus of RpoA subunits of the RNA polymerase, thereby acting both as a transcriptional repressor and activator: by binding to RpoA, Spx prevents binding of alternative transcription regulators to the RNA polymerase and in addition masks operator sequences of promoters, thereby acting as an anti-activator (Nakano *et al.*, 2003; Zuber, 2004). In contrast, a subset of promoters was found to be activated by RpoA-Spx interactions: Binding of Spx leads to conformational changes in the structure of RNA polymerase and is thereby altering its affinity to a motif upstream of the -35 region of activated promoters. Spx itself does not interact with DNA (Reyes and Zuber, 2008). Expression of Spx in *B. subtilis* is transcriptionally controlled by repressors the YodB and PerR, and by the sigma factors SigB and SigM (Petersohn *et al.*, 1999; Thackray and Moir, 2003; Leelakriangsak *et al.*, 2007). Most important, its expression is post-translationally regulated by proteolysis through protease ClpXP (Nakano *et al.*, 2002). Spx is an important pleiotropic regulator in all Gram-positive bacteria and has been shown to be also involved in stress tolerance and virulence in *S. mutans* or biofilm formation in *S. epidermidis* and other bacteria (Kajfasz *et al.*, 2010; Wang *et al.*, 2010).

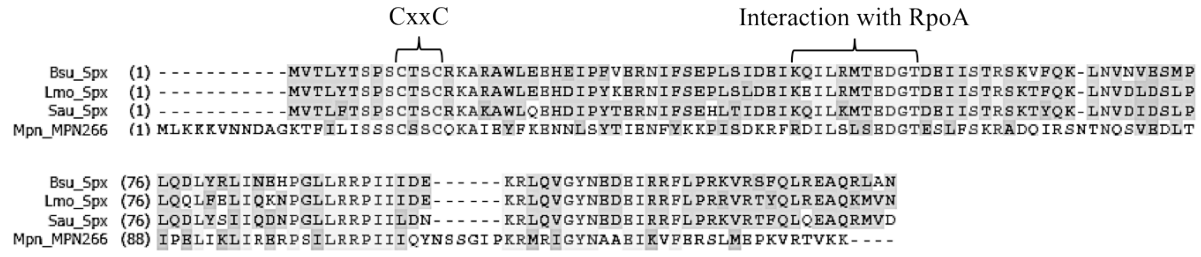


Fig. 1.5: Mpn266 is an Spx ortholog in *M. pneumoniae*

Multiple alignment of Spx from *B. subtilis* (Bsu*), L. monocytogenes* (Lmo), *S. aureus* (Sau) with Mpn266. The CxxC motif involved disulphide stress sensing and the domain required for interaction with RpoA are conserved in Mpn266.

A conserved CxxC motif involved in stress sensing and the domain required for interaction with RpoA in Mpn266 are conserved (see Fig. 1.5). In addition, Mpn266 was co-purified with the RNA polymerase in a global interactome study in *M. pneumoniae*, suggesting an analogous function in this bacterium (Kühner *et al.*, 2009). However, neither *cis*-acting transcription activators nor activator proteins that might interact with the RNA polymerase are known in *M. pneumoniae*, and DNA motifs of orthologous genes up-regulated by Spx in other bacteria are missing. Therefore, the role of Mpn266 in transcription regulation is not known to date.

Mpn241/WhiA family

Mpn241 is a protein encoded in an operon conserved in all Gram-positive bacteria. It is named after its orthologue WhiA in *Streptomyces coelicolor*, in which disruption of this gene leads to impaired sporulation, loss of autoregulatory transcription activation prior to sporulation and white colonies (Aínsa *et al.*, 2000). To date, *S. coelicolor* is the only organism in which a phenotype has been described. However, if the phenotype of WhiA deletion in *S. coelicolor* is due to unregulated transcription of WhiA controlled genes is not known.
In silico predictions show that WhiA proteins possess a helix-turn-helix like structure (Knizewski and Ginalski, 2007). A structural analysis of a WhiA ortholog from *T. maritima* confirmed the presence of a helix-turn-helix-domain that strongly resembles regions of bacterial sigma70 factors that bind to the -35 region of promoters. In addition, it could be shown that this domain is tethered to LAGLIDADG homing endonucleases scaffold, proteins so far only described in eukaryotes. Analysis of the putative catalytic domain shows that these proteins have lost their nuclease activity and only retained the ability to bind nucleic acids, either DNA or RNA (Longo *et al.*, 2005; Kaiser *et al.*, 2009). Thus, these proteins seem to be domesticated enzymes with eukaryotic ancestors that have been transformed to a new subgroup of proteins with different functions in an evolutionary process after horizontal gene transfer.

Mpn626

Open reading frame *mpn626* encodes a protein of unknown function. It was proposed to be a secondary sigma factor due to sequence and predicted structural homologies to *B. subtilis* SigD (Bornberg-Bauer and Weiner 3rd, 2002). However, it was not co-purified with the *M. pneumoniae* RNA polymerase in a global interactome study, even though it seems to be

associated with the ribosome (Kühner *et al.*, 2009; Anne-Claude Gavin, personal communication). Therefore, function of this protein remains to be unknown.

Mpn244/DisA

Open reading frame *mpn244* encodes a putative DisA orthologue from *B. subtilis*. *B. subtilis* DisA (DNA integrity scanning protein) forms an octamer that possesses unspecific DNA binding activity and moves rapidly along the DNA, pausing only at sites of DNA damage (Bejerano-Sagie *et al.*, 2006). Sporulation is delayed in a DisA-dependent manner until damaged DNA is repaired. In addition, DisA was shown to possess diadenylate cyclase activity, leading to formation of c-di-ATP which was proposed to be involved in signalling aiming to recruit the DNA repair machinery (Witte *et al.*, 2008). Furthermore, a protein with cyclic dinucleotide phosphodiesterase activity that hydrolyzes c-di-ATP and c-di-GTP involved in DNA damage recognition has been identified in *B. subtilis* (Rao *et al.*, 2009).
Secondary nucleotide messengers such as (p)ppGpp or cAMP are involved in transcriptional regulatory processes in other bacteria, and additional roles for the analogue messenger c-di-AMP were recently discovered. Therefore, a role of c-di-AMP in regulating gene expression cannot be excluded.

Mpn273/Hit

Open reading frame *mpn273* encodes a HIT orthologue conserved in all organisms. The protein contains a histidine triad (HIT) motif, His-x-His-x-His-x-x (with “x” being a hydrophobic amino acid) and is thought to be involved in cell cycle regulation (Séraphin, 1992).

Further proteins putatively involved in transcription regulation are Mpn397/RelA involved in the so called ´stringent response´, and the kinase/phosphatase couple Mpn223/HprK and Mpn247/PrpC that are involved in transcription regulation in other Gram-positive bacteria, a regulatory mechanism referred to as carbon catabolite repression. These two signalling pathways are the main scope of this work; therefore they will be discussed separately in the next chapter.
In addition to these proteins, a recent study revealed the presence of about 30 small non-coding RNAs in the genome of *M. pneumoniae*, none of which had been described so far (Güell *et al.*, 2009). Since these findings were published at the end of this work, their putative implications in regulation events are not further discussed here.

1.3 Mpn397/RelA and the ´stringent response´

One of the best-studied signalling pathways in bacteria is the stringent response, which is induced during nutrient starvation. It is characterized by the accumulation of the secondary nucleotide messengers or alarmones pppGpp and ppGpp (collectively referred to as (p)ppGpp, see Fig. 1.6) that result from the ATP-dependent phosphorylation of GTP and GDP, respectively, and by the down-regulation of stable RNA synthesis such as tRNA and rRNA.

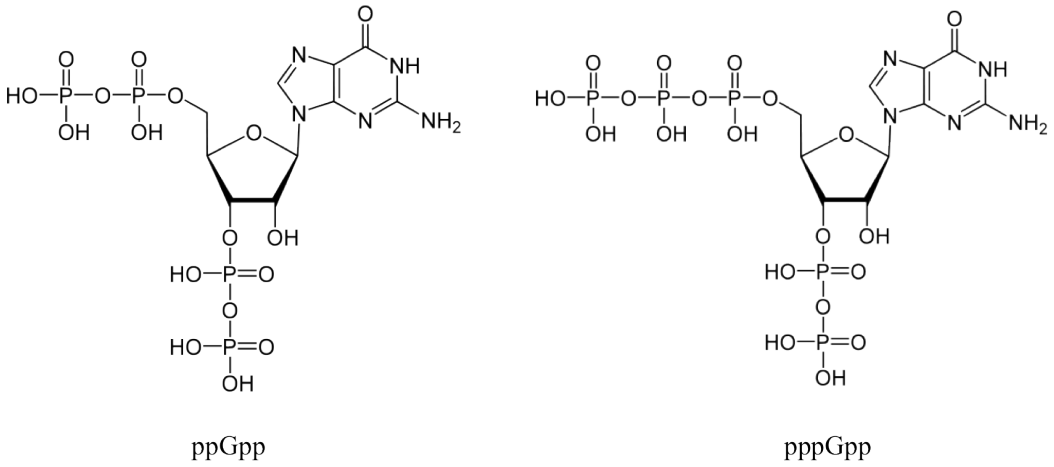


Fig. 1.6: ppGpp and pppGpp

Structures of the secondary nucleotide messenger molecules guanosin-5´-diphosphate-3´-diphosphate (ppGpp) and guanosin-5´-triphosphate-3´-diphosphate (pppGpp), collectively referred to as (p)ppGpp. Both are generated by the ATP-dependent phosphorylation of GDP and GTP, respectively.

The stringent response is a global regulatory mechanism shown to control transcription, translation, enzymatic activities and even the cell cycle. It is conserved among most bacteria and even in higher plants (van der Biezen *et al.*, 2000).

In Gram-negative bacteria such as *E. coli* in which the stringent response is described most exhaustively, two enzymes, RelA and SpoT, are responsible for the synthesis of (p)ppGpp. RelA is a (p)ppGpp synthetase that responds to amino acid starvation. Its activity is triggered by the interaction with ribosomal proteins in the presence of unloaded tRNAs (Haseltine *et al.*, 1973). The second emzyme, SpoT, functions both as a (p)ppGpp synthetase and hydrolase. Its synthethase activity is triggered by the interaction with the acyl carrier protein and thereby reflects fatty acid synthesis (Battesti and Bouveret, 2006; Battesti and Bouveret, 2009). In contrast, in *B. subtilis* and other Gram-positive bacteria only one enzyme is responsible for (p)ppGpp synthesis and hydrolysis under stringent conditions. To avoid any confusion, this enzyme will be referred to as RelA, even though a different name was proposed in recent publications (Potrykus and Cashel, 2008). In addition, small constitutively

active (p)ppGpp synthetases are present in most Gram-positive bacteria. However, they are not responsible for (p)ppGpp synthesis under stringent conditions and their expression is regulated in response to certain stresses, thereby triggering intracellular (p)ppGpp levels (Nanamiya *et al.*, 2008; Cao *et al.*, 2009).

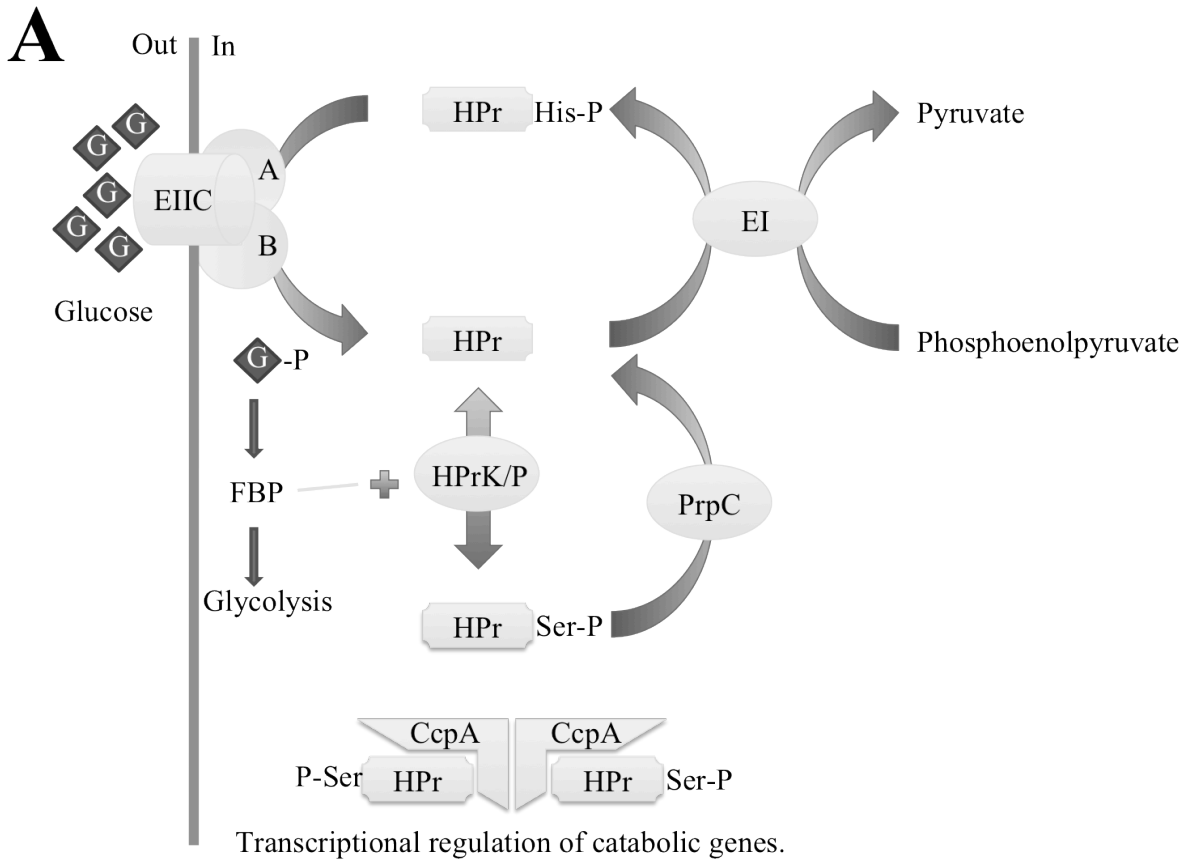
(p)ppGpp itself is a global regulator that controls both transcription and enzymatic activities. In *E. coli*, transcription regulation is mediated by the complex formation of (p)ppGpp with the protein DksA which interacts with the RNA polymerase, and by GC-rich discriminator sequences of certain promoters (Magnusson *et al.*, 2007; Wagner, 2002). In *B. subtilis* and other Gram-positive bacteria, no DksA is present and transcriptional changes are passive effects that are resulting from lowered intracellular GTP levels in stringent cells: In *B. subtilis*, (p)ppGpp inhibits the activity of IMP dehydrogenase, a key enzyme of GTP synthesis, thereby leading to lowered GTP levels that can be detected by transcription factors such as CodY (Pao and Dyess, 1981; Lopez *et al.*, 1981; Ochi *et al.*, 1982; Tojo *et al.*, 2008). More importantly, lowered intracellular GTP levels lead to a significant down regulation of transcription of genes initiating transcripts with GTP, and it was shown that changes of the initial base leads to a loss of down-regulation during stringent conditions (Krásny and Gourse, 2004; Tojo *et al.*, 2010). In addition to IMP dehydrogenase, (p)ppGpp was shown to inhibit various enzymes, and recently it was shown that (p)ppGpp dependent inhibition of DNA primase in stringent controlled cells leads to a stop in replication in both *E. coli* and *B. subtilis* (Wang *et al.*, 2007; Maciąg *et al.*, 2010).

Functioning as a global regulator that helps to respond to changing nutrient availability, (p)ppGpp is also involved in pathogenesis in several bacteria. For example, RelA proteins are required for the expression of the *virB* secretion system in *Brucella suis*, for surface attached growth and virulence of *Listeria monocytogenes* and for virulence of *Enterococcus faecalis* (see Dalebroux *et al.*, 2010 for more examples). Moreover, an almost unnoticed publication compared available microarray data of several facultative intracellular pathogens, thereby showing that the stringent response is commonly induced when these bacteria are entering their host cells (La *et al.*, 2008). This finding is supported by publications that show that RelA proteins are either strictly required for intracellular growth or to establish virulence in facultative intracellular pathogens (Dalebroux *et al.*, 2010). The absence of RelA proteins in eukaryotes, with the exception of higher plants, makes them ideal targets of novel antibiotics (Wexsellblatt *et al.*, 2010).

1.4 The phosphotransferase system (PTS) and carbon catabolite repression

Uptake of different carbon sources in bacteria is achieved either passively by diffusion or by active transport. Many carbon sources are imported by substrate-specific phosphotransferase systems (PTS), a family of transporters that couples uptake of the respective carbon source with its phosphorylation. The PTS in Gram-positive bacteria is best studied in the model organism *B. subtilis* (see Fig. 1.7 A; for a comprehensive overview about the PTS in bacteria, see Deutscher *et al.*, 2006): phosphate is transferred to a histidine residue (His15) of phosphocarrier protein HPr by PTS enzyme I, using phosphoenolpyruvate as phosphate donor. HPr(His15-P) then transports the phosphate to the PTS enzyme II complex located in the membrane, where glucose (or other carbon sources, depending on the PTS) is taken up and thereby phosphorylated to glucose-6-phosphate, the first intermediate of glycolysis. The unphosphorylated HPr can then again be loaded with a phosphate at the His15 residue by enzyme I. In addition to the phosphorylation of His15 by enzyme I, HPr can be phosphorylated at a serine residue Ser46 by the enzyme HPr kinase/phosphorylase, HPrK/P, using ATP as phosphate donor. Even though this enzyme both catalyzes the phosphorylation and dephosphorylation of Ser46, its kinase activity is enhanced by elevated intracellular levels of fructose-1,6-bisphosphate and ATP (Jault *et al.*, 2000; Ramström *et al.*, 2003). To prevent phosphorylation of all HPr at high intracellular levels of fructose-1,6-bisphosphate and ATP, a second enzyme was found to be involved in dephosphorylation of HPr-SerP, the protein phosphatase PrpC (Singh *et al.*, 2007). Both phosphorylation states of HPr are mutually exclusive, and HPr(Ser46-P) is a cofactor for the carbon catabolite control protein CcpA (Deutscher *et al.*, 1995). HPr(Ser46-P) forms a heteromeric complex with CcpA, thereby activating its DNA binding activity. The CcpA/Hpr(Ser46-P) complex then regulates transcription of catabolic genes by binding to the so called *cre* (catabolite repression element) sequence upstream of these genes (Miwa *et al.*, 2000). On the other hand, HPr(His15-P) can interact with and thereby activate transcriptional regulator YesS to activate transcription of genes required for pectin/rhamnogalacturonan utilization (Poncet *et al.*, 2009). In addition, HPr-HisP can transfer the phosphate from His15 to other protein such as the glycerol kinase GlpK or proteins involved in antitermination such as LicT, thereby triggering their enzymatic activity or their affinity towards their regulated target, respectively (Lindner *et al.*, 1999; Charrier *et al.*, 1997). Thus, the phosphorylation state of HPr helps the cell to monitor and to respond to the availability of different sugars, making HPr the key mediator of regulation in response to different carbon sources in *B. subtilis* and other *Firmicutes*.

In *M. pneumoniae*, four different PTS for glucose, fructose, mannitol and ascorbate are found, howevr only glucose and fructose were shown to promote growth (Halbedel *et al.*, 2004; Yus *et al.*, 2009; this work). The main mechanisms in phosphate transfer in *M. pneumoniae* are the same as in *B. subtilis* (see Fig 1.7 B). However, a characterisation of HPrK/P from *M. pneumoniae* showed that this enzyme is mainly active as a kinase due to an elevated affinity of the enzyme towards ATP (Steinhauer *et al.*, 2002). The finding of contrary phosphorylation states of HPr in *M. pneumoniae* mutants lacking HPrK and PrpC supported this finding, suggesting monofunctional and antagonistic roles of HprK and PrpC in HPr phosphorylation (Halbedel *et al.*, 2004; Halbedel *et al.*, 2006). Although these two enzymes tightly control the phosphorylation state of HPr, no regulatory elements such as antiterminator proteins or transcriptional regulators described in other bacteria are found in *M. pneumoniae*. In addition, a recent phosphoproteome analysis of an HPrK mutant showed that HPr is the only substrate of HprK *in vivo*, ruling out additional roles of this enzyme in this organism (Schmidl *et al.*, 2010). These findings raise the question about the role of this conserved mechanism in *M. pneumoniae*, which is so far unknown.



Transcriptional regulation of catabolic genes.

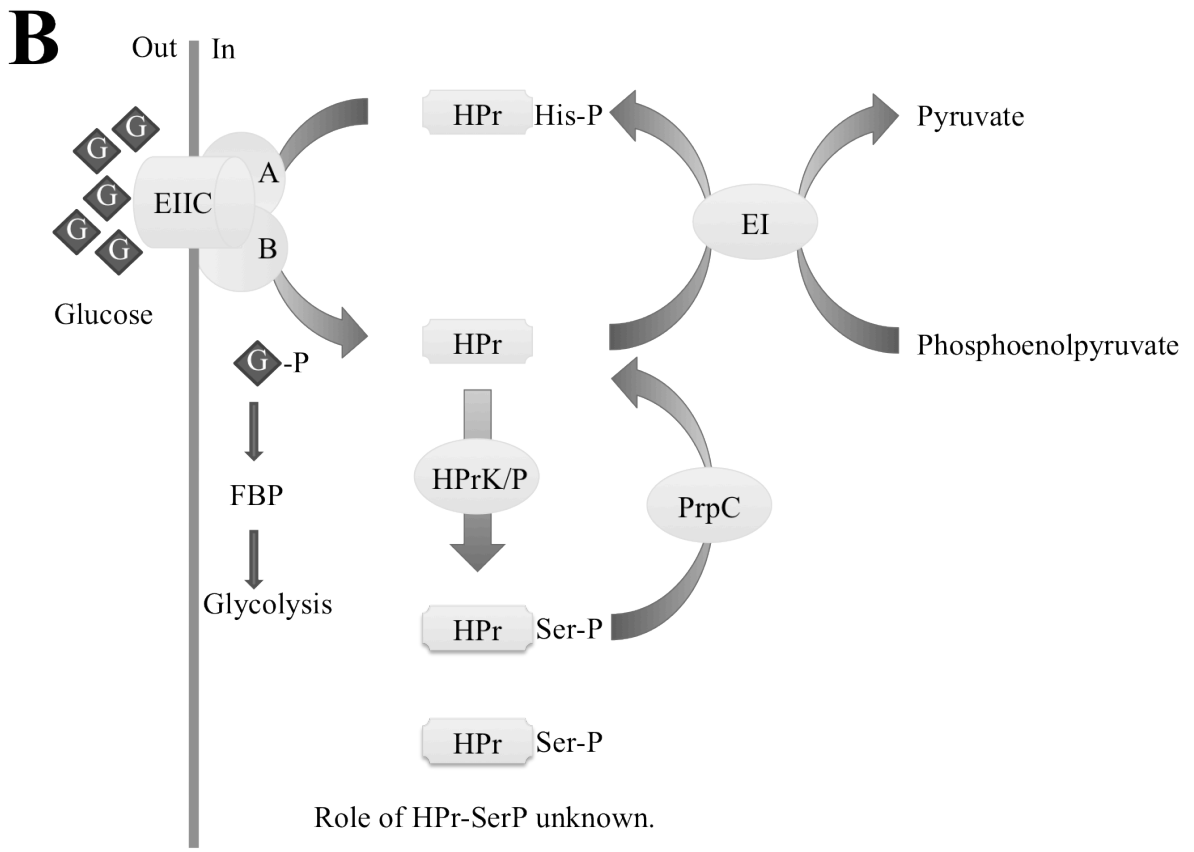


Fig. 1.7: Comparison of the PTS in *B. subtilis* (A) and *M. pneumoniae* (B).

The mechanisms of HPr phosphorylation are conserved in *M. pneumoniae*. However, the function of HPr(Ser46-P) in this organism is so far unknown.

1.5 Aims of this work

During its reductive evolution, *M. pneumoniae* not only reduced its metabolic potential and its size, but also its genetic requirements to sense and to react to environmental changes. This raises the questions if a minimal genome only requires a minimum of regulation, and which processes in particular need to be regulated in a minimal cell. Until recently, *M. pneumoniae* and other *Mollicutes* were thought to regulate transcription only by very few, rather simple mechanisms. In contrast to this concept, the work of Güell *et al.* (2009) suggested surprisingly complex changes in the transcriptome in response to environmental changes and stresses. However, the work of Güell *et al.* did neither reveal any mechanism how transcription is regulated, nor if the transcriptional changes observed are physiologically relevant.

The main aim of this work was to find target genes under control of the so far uncharacterized transcription regulators and/or to identify additional genes that are involved in transcription regulation in *M. pneumoniae*.

2. Materials and methods

2.1 Microbiological techniques

Materials: Chemicals, utilities, equipment, commercial systems, proteins, antibodies, enzymes, oligonucleotides, software and websites are listed in the appendix of this work.

2.1.1 Bacterial strains and plasmids

A list of all bacterial strains and plasmids used in this study can be found in the appendix of this work.

2.1.2 Growth media and facultative additives

All buffers, solutions and media were prepared with deionized water and autoclaved (20 min at 121°C and 2 bar). Thermolabile substances were dissolved and sterilized by filtration. All solutions are prepared with water; other solvents are indicated if used. Final volumes are indicated. For solid media, 15 g l^{-1} agar was supplemented.

Media used to grow *Escherichia coli* and *Bacillus subtilis*

LB medium (1 l)	Tryptone	10 g
(Bertani, 1951)	Yeast extract	5 g
	NaCl	10 g
5x C salts (1 l)	KH_2PO_4	20 g
	K_2HPO_4 x 3 H_2O	80 g
	$(NH_4)_2SO_4$	16.5 g
III´salts (1 l)	$MnSO_4$ x 3 H_2O	0.232 g
	$MgSO_4$ x 7 H_2O	12.3 g

10x MN medium (1 l)	K_2HPO_4 x 3 H_2O	136 g
	KH_2PO_4	60 g
	Sodium citrate x 2 H_2O	10 g
1x C minimal medium	5x C salts	20 ml
(100 ml)	Tryptophan (5 mg ml^{-1})	1 ml
	Ammonium iron citrate	1 ml
	(2.2 mg ml^{-1})	
	III´ salts	1 ml
1x CS medium with glucose	5x C salts	20 ml
(100 ml)	Tryptophan (5 mg ml^{-1})	1 ml
	Ammonium iron citrate	1 ml
	(2.2 mg ml^{-1})	
	III´ salts	1 ml
	Sodium succinate (30%)	2 ml
	Glucose (20%)	2.5 ml
SP medium (1 l)	Nutrient Broth	0.8 g
	$MgSO_4$ x 7 H_20	0.25 g
	KCl	1 g
	H_20	ad 1 l
	(autoclave, after cooling addition of:)	
	$CaCl_2$ (0.5 M)	1 ml
	$MnCl_2$ (10 mM)	1 ml
	Ammonium iron citrate	2 ml
	(2.2 mg ml^{-1})	

MNGE medium (10 ml)	1x MN medium	8.77 ml
	Glucose (20%)	1 ml
	Potassium glutamate (40%)	50 µl
	Ammonium iron citrate (2.2 mg ml^{-1})	50 µl
	Tryptophan (5 mg ml^{-1})	100 µl
	$MgSO_4$ x 7 H_2O (1 M)	30 µl
	+/- CAA (10%)	100 µl
Starch agar plates (1 l)	Nutrient broth	7.5 g
	Starch	5 g
	Agar	15 g

Media used to grow of *Mycoplasma pneumoniae*

MP medium (500 ml)	PPLO broth	7.35 g
Modified Hayflick medium	HEPES	11.92 g
(Chanock et al., 1962)	Phenol red (0.5%)	2 ml
	Sodium hydroxide (2 N)	14 ml
	(addition of water, adjust pH to 7.6 – 7.8 and autoclave; after cooling add the following:)	
	Horse serum (heat inactivated)	100 ml
	Penicillin (100 000 U ml^{-1})	5 ml
	Glucose or glycerol (50%)	10 ml

The medium later referred to as ´**Hayflick light**´, is based on the same composition as described above, but lacks phenol red and horse serum to reduce signal background in LC/MS experiments. To get the same volume, water was used instead.

Defined medium		Reagent	Final concentration
MM14	**Basal solution**	Na_2HPO_4	2 mM
(Yus *et al.*, 2009)		NaCl	100 mM
		KCl	5 mM
		$MgSO_4$	0.5 mM
		$CaCl_2$	0.2 mM
	Carbon sources	Glucose	10 g l^{-1}
		Glycerol	0.5 g l^{-1}
	Vitamins	Spermine	0.1 mM
		Nicotinic acid	1 mg l^{-1}
		Thiamin	1 mg l^{-1}
		Pyridoxamine	1 mg l^{-1}
		Thioctic acid (lipoamide)	0.2 mg l^{-1}
		Riboflavin	1 mg l^{-1}
		Choline	1 mg l^{-1}
		Folic acid	1 mg l^{-1}
		Coenzyme A	1 mg l^{-1}
	Bases	Guanine/Cytidine/Adenine	20 mg l^{-1}
	Lipids	Cholesterol	20 mg l^{-1}
		Palmitic acid	10 mg l^{-1}
		Oleic acid	12 mg l^{-1}
		BSA (fatty acid free)	2 g l^{-1}
	Amino acids	Cysteine	2 mM
		Peptone	2.5 g l^{-1}
	Others	HEPES	50 mM
		Phenol red	2 mg l^{-1}
		Penicillin	1000 U ml^{-1}

The medium was prepared by mixing concentrated stock solutions of the different compounds (indicated below). Stocks were stored at -20°C until used. Except for the lipids that were solved in ethanol, all stock solutions were prepared with deionized water. Lipids were mixed with BSA as a carrier when added. After mixing the compounds, the pH was adjusted to 7.7 – 7.8, the medium was sterilized by filtration and stored at 4°C.

Compound	**Concentration**
Basal	5x
MgSO4	2000x
CaCl2	4000x
Peptone 5%	20x
Cysteine 100 mM	50x
Glucose 50%	50x
Glycerol 5%	100x
Cytidine/Guanine/Adenine	100x
Vitamins	100x
Coenzyme A	1000x
Lipids (serum substitute)	5x
HEPES 1M	20x

Medium used for human cell cultures (HeLa and A549 cells)

DMEM (Dulbecco's modified Eagle's medium, Dulbecco and Freeman, 1959)	GIBCO DMEM (high glucose, with pyruvate)	90 ml
	Fetal calf serum	10 ml
	L-glutamine (1 mM)	200 µl
	Penicillin (100 000 U ml^{-1})	500 µl

Antibiotics

All antibiotics were prepared as 1000x concentrated solutions. Except for erythromycin, which was solved in 70% ethanol, all antibiotics were solved in deionized water. The solutions were sterilized by filtration and stored at -20°C. When used, solutions were thawed on ice and added to the fresh, autoclaved medium after it was cooled down to about 50°C. For

the different organisms, the following final concentrations have been used.

E. coli	Ampicillin	100 µg ml^{-1}
	Kanamycin	50 µg ml^{-1}
B. subtilis	Chloramphenicol	5 µg ml^{-1}
	Kanamycin	5 µg ml^{-1}
	Erythromycin*	2 µg ml^{-1}
	Lincomycin*	25 µg ml^{-1}
	Spectinomycin	100 µg ml^{-1}
	*for selection for *ermC*, a mixture of erythromycin and lincomycin was used in their respective concentration, see above.	
M. pneumoniae	Gentamycin (liquid media)	80 µg ml^{-1}
	Gentamycin (solid medium)	160 µg ml^{-1}
	Penicillin	1000 U ml^{-1}

X-Gal (5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid)

A stock solution of 40 mg ml^{-1} was prepared; the final concentration in solid media was 40 µg ml^{-1}. DMF (dimethylformamide) was used as solvent, and the solution was stored in darkness at -20°C.

2.1.3 Cultivation techniques

Cultivation and storage of *E. coli*

Unless otherwise stated, *E. coli* was grown in LB medium at 37°C and 200 rpm in glass tubes or flasks. Cultures were inoculated with single colonies on fresh agar plates or from overnight cultures. Growth was monitored by OD measurement at λ = 600 nm.

E. coli was stored on LB agar plates at 4°C for up to 4 weeks. For long-term storage, aliquots of fresh cultures were mixed with glycerol or DMSO to a final concentration of 15% and 10%, respectively, chilled on ice and than frozen and stored at -80°C.

Cultivation and storage of *B. subtilis*

B. subtilis was grown in LB medium, CS glucose medium or MNGE medium at 37°C and 200 rpm in glass tubes or flasks. Cultures were inoculated with single colonies on fresh agar plates, with spores from SP agar plates or from overnight cultures. Growth was monitored by OD measurement at λ = 600 nm. For faster growth on solid medium, the agar plates were incubated at 42°C.

B. subtilis was stored on LB medium at room temperature not longer than 3 days. For long-term storage, *B. subtilis* was grown on SP agar plates and stored at room temperature, or frozen stock cultures with glycerol were prepared as described for *E. coli*.

Cultivation and storage of *M. pneumoniae*

Unless stated otherwise, *M. pneumoniae* was grown at 37°C in modified Hayflick medium for 96 h in culture flasks. Culture flasks with 12.5, 75, 150 and 300 cm^2 surface with 10, 50, 100 and 200 ml modified Hayflick medium were used. Cultures were inoculated with 1 ml stock culture per 100 ml medium.

For long-term storage of *M. pneumoniae*, the supernatant of a fresh culture was discarded after 96h and cells were scraped in 20 ml fresh modified Hayflick medium. Aliquots of 10 ml were frozen and stored at -80°C.

Cultivation and storage of human cell lines (HeLa, A549)

Human cell lines were grown in DMEM medium at 37°C and 5% CO_2 in culture flasks. After 4 days of growth the medium of a confluent culture was discarded, cells washed twice with PBS and incubated with 500 µl trypsin/EDTA at 37°C for up to 5 minutes. When all cells were detached, the trypsin treatment was stopped by the addition of 15 ml fresh DMEM. The cell suspension was transferred into a sterile falcon tube and cells were pelleted by centrifugation (1000 rpm, 10°C, 5 minutes). The supernatant was discarded, and the pellet was diluted in 10 ml fresh DMEM. Viability was controlled by microscopy and life/death staining using trypan blue. 200 µl of the suspension were used for the inoculation of a 25 cm^2 culture flask containing 25 ml DMEM.

Cell lines were grown for a maximum of 10 passages and then fresh cells from frozen stocks were used to start new cultures.

Cell density was determined by using a Neubauer chamber (0.1 mm depth, 0.0025 mm surface). 10 µl cell suspension were gently mixed with 10 µl trypan blue and incubated for 3 min. 10 µl of the stained cells were transferred into the space between the cover slip and the

Neubauer chamber. Living cells within 4 big squares (consisting of 16 smaller squares each) were counted. The number of cells per ml was calculated by the multiplication of the average number of cells per big square by the dilution factor 2 and the volume factor 1 x10^4.

$$Cells/ml = \frac{\Sigma cells}{4} \times 2 \times 10^4$$

2.2 Molecular biology and biochemical techniques

2.2.1 General methods

A summary of standard methods used during this study is given, together with its relevant publication, in table 2.1 below.

Method	**Reference**
Absorption measurement	Sambrook *et al*., 1989
Ethidiumbromide staining of nucleic acids	Sambrook *et al*., 1989
Precipitation of nucleic acids	Sambrook *et al*., 1989
Gel electrophoresis of DNA	Sambrook *et al*., 1989
Ligation of DNA fragments	Sambrook *et al*., 1989
Determination of Protein concentration	Bradford, 1976
Denaturating gel electrophoresis of proteins	Laemmli, 1970
DNA Sequencing by Sanger *et al.*	Sanger *et al.*, 1977

Tab. 2.1: Standard methods used in this work and their corresponding Publication

2.2.2 Genetic manipulation of *E. coli*, *B. subtilis* and *M. pneumoniae*

Preparation of competent of *E. coli*

Two different methods were used to prepare competent *E. coli* cells:

I. Method: $MnCl_2$

4 ml LB medium were inoculated with a single colony of *E. coli* and incubated at 37°C and 200 rpm overnight. This preculture was used to inoculate 250 ml SOB medium. The culture

was grown for up to 24 h at 18°C and 200 rpm until an OD_{600} of 0.5 to 0.9 was reached. The culture was chilled on ice for 10 min and then centrifuged (10 min, 5000 U min^{-1}, 4°C). The supernatant was discarded and the pellet dissolved in 80 ml ice-cold TB buffer and again incubated on ice for 10 min. Cells were sedimented by centrifugation (10 min, 5000 U min^{-1}, 4°C) and the pellet suspended in 18.6 ml ice-cold TB buffer. Quickly, 1.4 ml of DMSO was added. Aliquots of 200 µl were transferred into sterile Eppendorf cups, frozen in liquid nitrogen and stored at -80°C until used.

SOB medium (1 l)	Trypton	20 g
	Yeast extract	5 g
	NaCl	0.584 g
	KCl	0.188 g
	(add 980 ml water, autoclave, then add)	
	$MgCl_2$ (1 M)	10 ml
	$MgSO_4$ (1 M)	10 ml
TB buffer (500 ml)	PIPES	1.51 g
	$CaCl_2$ x H_2O	1.1 g
	KCl	9.32 g
	(add 472 ml water, autoclave, then add)	
	$MnCl_2$ (1 M)	28 ml
	pH 6.7	

II. Method: $RbCl_2$

500 ml of LB medium were inoculated with 4 ml preculture (prepared as described in method I). The culture was grown at 37°C and 200 rpm for 4 hours in a baffled flask. Shaking in ice water chilled the culture and cells were then sedimented by centrifugation (10 min, 5000 U min^{-1}, 4°C). The pellet was suspended in 20 ml ice-cold TfbI. Cells were centrifuged (10 min, 5000 U min^{-1}, 4°C) , the supernatant discarded and the pellet suspended in 4 ml ice-cold TfbII. Aliquots of 200 µl were frozen in liquid nitrogen and stored at -80°C until used.

Buffer TfbI (100 ml)	Potassium acetate	0.29 g
	$MnCl_2$	0.99 g
	$RbCl_2$	1.21 g
	$CaCl_2$	0.147 g
	Glycerol	30 ml
	pH 5.8	
Buffer TfbII (100 ml)	MOPS	0.21 g
	$CaCl_2$	1.1 g
	$RbCl_2$	0.121 g
	Glycerol	30 ml
	pH 6.8	

Transformation of competent *E. coli*

For the transformation of competent *E. coli*, the aliquots were gently thawed on ice. Cells were mixed with 1 µl of a 1/10 dilution of a high-copy plasmid preparation, 5 µl of a low-copy plasmid preparation or 10 µl of a plasmid ligation sample and incubated on ice for 20 min. Then, the samples were incubated at 42°C for 2 min, chilled on ice for 5 min and mixed with 800 µl LB medium. After growth at 37°C and 200 rpm for 1 h, 100 µl of the samples were streaked on LB selection plates and the rest sedimented by centrifugation. The supernatant was discarded, the pellet suspended in 100 µl LB and plated on a separate LB selection plate. The plates were incubated overnight at 37°C. Every time this experiment was performed, a negative control without added DNA was performed in parallel.

Preparation of competent *B. subtilis*

An overnight culture was used to inoculate 10 ml MNGE medium containing 1% casamino acids (CAA) to an OD_{600} of 0.1 and incubated at 37°C and 200 rpm. Growth was monitored by following the OD_{600}. When an OD_{600} of 1.3 was reached, 10 ml MNGE medium without CAA were added to dilute the culture. After another incubation at 37°C and 300 rpm for 1 h, the cells were centrifuged (5000 rpm, 5 min at room temperature), the supernatant transferred into a sterile tube and the pellet suspended in 2.5 ml of the supernatant. Cells could directly be used for transformation (400 µl aliquots per transformation sample) or were mixed with glycerol to a final concentration of 10%, frozen in liquid nitrogen and stored at -80°C for later use.

Transformation of competent *B. subtilis*

Aliquots of frozen competent *B. subtilis* cells were thawed at 37°C and supplemented with 100 µl of the following solution:

MN (1x)	1.7 ml
Glucose (20%)	43 µl
$MgSO_4$ (1 M)	34 µl

About 5 µg of plasmid DNA or a whole LFH-PCR sample was added to the cells and the sample was incubated at 37°C and 200 rpm for 30 min. Meanwhile, a nutrient rich expression solution was prepared:

Expression solution	Yeast extract (5%)	50 µl
(105 µl)	CAA (10 %)	25 µl
	Tryptophan (5 mg ml^{-1})	5 µl
	Water	25 µl

The transformation sample was supplemented with the expression solution and incubated for another hour at 37°C and 200 rpm. After that, the cells were spread on SP or LB plates containing the selective antibiotic.

Targeted mutagenesis of *B. subtilis*

Targeted knockout of genes in *B. subtilis* was achieved by homologous recombination. Competent *B. subtilis* cells were either transformed with chromosomal DNA containing the desired mutation to obtain isogenic strains, or with DNA fragments that contained a resistance cassette flanked by the neighbouring regions (+/- 1 kbp) of the targeted gene. Such fragments were generated by long-flanking homology PCR (LFH-PCR, see chapter 2.2.3 for a detailed protocol).

Transformation of *M. pneumoniae*

M. pneumoniae lacks the genomic background to develop competence. Therefore it can only be transformed by electroporation. Since no plasmid or protocol for homologous recombination is available for *M. pneumoniae*, genetic manipulation can only be done by the use of transposons. Resistance towards gentamycin was the only marker for *M. pneumoniae*

mutants used in this study. Since spontaneous resistance towards gentamycin occurs randomly, a negative control was always prepared together with the transformation sample to monitor the spontaneous mutation rate.

A 100 ml culture of *M. pneumoniae*, sufficient for 3 to 4 transformations, was grown for 96 h at 37°C in Hayflick medium. The medium was discarded and adherent cells were washed twice with ice-cold electroporation buffer. Then, cells were scraped in 1.5 ml of electroporation buffer, sedimented by centrifugation (5 min, 10000 rpm, 4°C) and the pellet again suspended in 15 µl of electroporation buffer.

50 µl of cells were transferred into an electroporation cuvette (0.2 cm space, pre-incubated on ice) and mixed with 8-10 µg DNA and 1 µl yeast tRNA (500 µg μl^{-1}). The mixture was brought to a final volume of 80 µl with electroporation buffer and incubated on ice for 15 min. After electroporation (2.5 kV, 25 µF, 100 Ω), the sample was incubated for another 15 min on ice. The sample was mixed with 4 ml MP medium with glucose (but without gentamycin) and incubated at 37°C for 4 h.

To get a mixed culture of random transposon insertion mutants, 1 ml of this sample was transferred to a culture flask (12.5 cm^2), brought to final volume of 10 ml with Hayflick medium containing gentamycin and incubated at 37°C until a colour change from red to yellow was visible.

To get single colonies, the cells were sedimented by centrifugation (5000 rpm, 5 min, room temperature), the pellet suspended in 1 ml Hayflick by using a syringe (∅ 0.1 mm) and a dilution series (1:10 steps) was prepared. 250 µl from dilutions 1^{-4} to 1^{-10} were plated on MP medium plates containing 160 µg ml^{-1} gentamycin, the plates dried for 30 min and incubated at 37°C and elevated humidity. After about 2 weeks, single colonies were visible and could be used to inoculate 1.5 ml Hayflick medium containing gentamycin. Cells were seeded in higher volumes as soon as a colour change from red to yellow was visible.

Electroporation buffer (1 l)	HEPES	8 mM
	Sucrose	272 mM
	pH 7.4	

Targeted mutagenesis of *M. pneumoniae*: Haystack mutagenesis

The only tool to manipulate *M. pneumoniae* are transposons, genetic elements that insert randomly but stably in the genome. To isolate mutants with a transposon insertion in the gene of interest (*goi*) from a mixture of random insertions, Haystack mutagenesis was used

(Halbedel *et al.*, 2006). The principle of this method is that about 3000 single random transposon insertion clones are grouped to pools of 50 clones. To identify clones with a desired transposon insertion, these pools are screened by PCR where primer pairs specific for the gene and for the transposon are used (couples A/C and B/D in figure 2.1). Once a positive pool is identified it is sub-screened with the same primer couples to identify the individual positive clone. This method allows the isolation of desired mutants affected in non-essential genes with a probability of 99.999%.

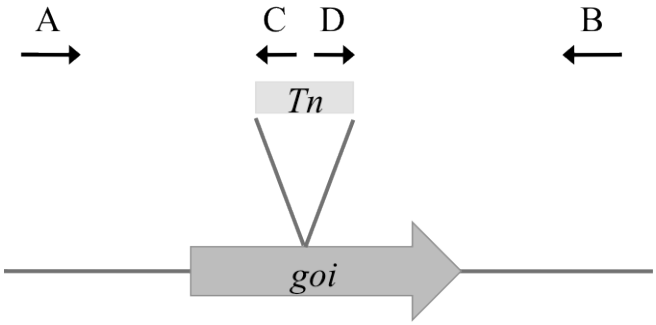


Figure 2.1: Principle of Haystack mutagenesis.
Individual mutants harbouring a transposon (*Tn*) insertion within a gene of interest (*goi*) can be identified and isolated from a pool of several random transposon mutants by PCR using primer pairs specific for the gene (A and B) and for the transposon (C and D).

Sequencing of the PCR products can then identify the exact insertion site. Identified clones are grown and further investigated by Southern blot analysis for single or multiple transposon insertions and to confirm gene disruption.

2.2.3 Experiments with DNA

For the work with DNA, only DNase-free solutions and materials were used. Unless mentioned otherwise, work was performed at room temperature, and DNA was solved in deionized water. Chromosomal DNA was stored at 4°C. Plasmids, oligonucleotides, PCR products and other fragmented DNA were stored at -20°C and thawed on ice when used. DNA concentrations were measured with a ND-1000 NanoDrop (Peqlab, Erlangen, Germany).

Isolation of chromosomal DNA from *B. subtilis* and *M. pneumoniae*

Genomic DNA from *B. subtilis* and *M. pneumoniae* was isolated using the DNeasy Tissue Kit (Qiagen, Hilden, Germany).

B. subtilis was grown overnight in LB medium at 37°C and 200 rpm. 1.5 ml of the culture was harvested by centrifugation (13.000 rpm, 2 min, room temperature), the pellet suspended in 180 µl lysis buffer and incubated at 37°C for 60 min. Further steps were performed according to the manufacturers instructions.

M. pneumoniae was grown in Hayflick medium (100 ml, 150 cm^2 flask) at 37°C for 96 h. The medium was discarded and adherent cells were washed twice with PBS. Cells were scraped in 1 ml PBS, centrifuged (13.000 rpm, 5 min, room temperature) and suspended in 540 µl lysis buffer (for simplicity only; lysozyme is not required since *M. pneumoniae* doesn't have a cell wall). 180 µl of the sample were directly used to isolate chromosomal DNA according to the manufacturers instructions, the rest was stored at -20°C for later use.

Preparation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* by using the Mini Prep Kit (Macherey-Nagel, Düren, Germany). *E. coli* carrying the desired plasmid was grown overnight in 4 ml LB medium containing a selective antibiotic at 37°C and 200 rpm. Cells were harvested by centrifugation (13.000 rpm, 2 min at room temperature) and the pellet used to isolate the plasmid according to the manufacturers instructions or stored at -20°C for later use.

DNA sequencing

Sequencing was done based on the chain termination method (Sanger *et al.*, 1977) with fluorescence labeled dideoxynucleotides. SeqLab (Göttingen, Germany) and the Laboratorium für Genomanalyse (G2L) of the Georg-August-University Göttingen performed the sequencing reactions. The sequences were analyzed with the LaserGene package (DNASTAR, Madison, Wisconsin, USA).

Gel electrophoresis and ethidium bromide staining

According to the expected size of the DNA fragments, 1% to 2% agarose gels in TAE buffer were used for analytical and preparative separation. To facilitate loading and to monitor migration of the samples in the gel, DNA samples were mixed with 5x DNA loading dye. Gels were run at 80-120 V until the xylencyanol color marker reached the lower third of the gel. After the run was finished, the gels were incubated in ethidium bromide solution for

5 min and then briefly rinsed with water. Visualisation and documentation was performed with a UV transilluminator 2000 (Bio-Rad, Munich, Germany) by exposing the gels to UV light (λ = 254 nm). The size of DNA fragments in the gel was estimated by comparison with λ DNA (digested with restriction enzymes EcoRI and HindIII) or with commercial DNA markers.

If DNA fragments should be isolated from the gel, UV exposure was performed at λ = 365 nm wavelength to prevent GC cross-linking. Desired bands were excised from the gel with a DNA-free scalpel and DNA was isolated with the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer´s instructions.

Southern blot analysis

Southern blots were used to validate single transposon insertions in the genome of *M. pneumoniae* mutants. An RNA probe that targets the gentamycin resistence cassette introduced by the transposon was used. Chromosomal DNA from *M. pneumoniae* wild type served as a negative control.

10 µg chromosomal DNA were digested overnight with restriction enzyme BglII, separated in a 1% agarose gel by electrophoresis and stained with ethidium bromide as described above. Transfer of DNA fragments from the gel onto a positively charged nylon membrane (Roche) was performed with a vacuum blot device (VavuGene^{TM} XI). 12 x 11 cm nylon membrane were moistened with deionized water, placed on the blotting device and covered with a plastic foil. The foil helps to establish the vacuum by sealing the surface not covered by the gel. The agarose gel was put onto the foil and a vacuum of 80 mbar was established. 10 ml of the following buffers were consecutively loaded onto the gel and incubated as indicated:

1. Depurination buffer	15 min
2. Denaturation buffer	20 min
3. Neutralisation buffer	20 min
4. 20x SSPE	5 h

The remaining buffer was discarded before the next buffer was added. After transfer, the DNA was cross-linked to the nylon membrane with UV light (λ = 254 nm) and stored dry and clean until further usage.

The nylon membrane was incubated with 20 ml pre-hybridisation buffer at 68°C in a hybridisation oven for 60 min. Then the pre-hybridisation buffer was replaced with hybridisation buffer (5 µl DIG-labelled RNA probe solved in 15 ml hybridisation buffer) and incubated overnight at 68°C in the hybridisation oven. The next day, the hybridisation buffer was transferred into a falcon tube and stored at -20°C until further usage. The membrane was washed twice for 15 min with 50 ml buffer 1 and twice with 50 ml buffer 2, each at room temperature, to get rid of unbound RNA probes.

Detection of DNA-RNA hybrids on the membrane followed directly after the hybridisation step. All steps were performed at room temperature and with gentle shaking. The membrane was incubated with buffer DIG-P1 for 5 min, then for 30 min with buffer DIG-P2. Then, anti-DIG antibodies coupled with alkaline phosphatase dissolved in buffer DIG-P2 (1:10000) were added onto the membrane and incubated for 30 min. After this, the membrane was washed and equilibrated three times 5 min with buffer DIG-P3. For visualisation of DNA-RNA hybrids, the membrane was put between clear plastic foil and incubated with 5 µl CDP* (diluted in 1 ml buffer DIG-P3) for 5 min. CDP* is a substrate of the alkaline phosphatase coupled to the anti-DIG antibody, resulting in chemo-luminescence as a coproduct which can be visualized and quantified. Depending on the intensity of the chemo-luminescence, the membrane was exposed for 14 – 45 min in a Chemi-Smart^{TM} 5000 luminescence system (PEQLAB, Erlangen, Germany).

PCR techniques: qPCR, PCR, LFH, and CCR

Reactions were prepared in PCR µl reaction tubes tubes in a final volume of 50 µl. Personal Thermocyclers (Biometra, Germany) or Labyclers (SensoQuest, Göttingen, Germany) were used. PCR products were purified either by agarose gel extraction or directly by using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Reagents and PCR products were stored at -20°C and thawed on ice when used.

Oligonucleotides

Specific oligonucleotide primers (here referred to as primers) were designed to amplify DNA fragments of interest. Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany) or from Sigma-Aldrich (Munich, Germany). Mutagenesis primers used for CCR (combined chain reaction) are 5´ phosphorylated. If the DNA fragment should be cloned into

a plasmid vector, recognition sequences for restriction enzymes were added via primers at both ends of the PCR product.

The annealing temperature of a PCR reaction has to be 2-5°C lower than the melting temperature of the primers used. The melting temperature *Tm* of a primer depends on its length and G/C content and can be calculated with the given formula, where %G+C represents the GC content of the primer and *n* the number of nucleotides:

$$Tm[^{\circ}C] = 69.3 + 0.41 \times (\%G + C) - \frac{650}{n}$$

All primers used in this work are listed in the appendix (chapter 9.2).

qPCR (quantitative PCR)

qPRC was used to validate enrichment of specific DNA fragments in SPICE experiments (see chapter 2.5). All primers used for qPCR have a melting temperature of 60°C and were designed to amplify a DNA fragment of 150 bp size. qPCR was performed in an iCycler (Biorad) by using the iScript™ One-Step Kit with SYBR® Green (Biorad) according to the manufacturers instructions except for the following modifications: Since DNA was used as a template, no reverse transcriptase was added to the reaction and the reverse transcription step was skipped in the PCR program. Instead, the program started with the Taq activation step (5 min, 95°C). 5 ng and 10 ng of fragmented, co-purified DNA were used as template in the reactions.

Polymerase chain reaction (PCR)

Different enzymes were used for various applications: for check PCR to identify positive clones carrying desired plasmids or screening for *M. pneumoniae* mutants, the thermo-stable DNA polymerase from *Thermus aquaticus* (*Taq*) was used. *Taq* polymerase synthesizes DNA with a speed of 100bp/sec at 72°C (Lawyer *et al.*, 1993), does not possess proof-reading activity and is known to statistically create one mismatch per 9000 bp (Tindall and Kunkel, 1988). Therefore, *Phusion*™ DNA Polymerase (Finnzymes, Espoo, Finland), an engineered DNA polymerase from *Pyrococcus furiosus* with enhanced proofreading activity, was used if the PCR product should be cloned in a plasmid vector.

PCR with *Taq* DNA polymerase (50 µl reaction)	
Template	10 ng plasmid DNA or 100 – 200 ng chrom. DNA
10x *Taq* reaction buffer	5 µl
dNTPs 12.5 mM	2 µl
Forward primer (20 pmol)	2.5 µl
Reverse primer (20 pmol)	2.5 µl
Taq DNA polymerase	1 µl
Deionized H_2O	Ad. 50 µl

PCR standard program for *Taq* DNA polymerase			
Initial denaturation	95°C	5 min	
Denaturation	95°C	30 sec	
Annealing	As calculated	45 sec	**30x**
Elongation	72°C	1 min/kb	
Final elongation	72°C	5 min	
Hold	15°C	∞	

PCR with *Phusion*™ DNA polymerase (50 µl reaction)	
Template	10 ng plasmid DNA or 100 – 200 ng chrom. DNA
5x *Phusion*™ reaction buffer	10 µl
Phusion™ dNTPs 10 mM	1 µl
Forward primer (20 pmol)	2.5 µl
Reverse primer (20 pmol)	2.5 µl
Phusion™ DNA polymerase	1 µl (1 U)
Deionized H_2O	Ad. 50 µl

PCR standard program for *Phusion*™ DNA polymerase			
Initial denaturation	98°C	1 min	
Denaturation	98°C	10 sec	**30x**
Annealing	As calculated	30 sec	
Elongation	72°C	20 sec/kb	
Final elongation	72°C	3 min	
Hold	15°C	∞	

Long flanking homology PCR (LFH-PCR)

LFH-PCR (Wach, 1996) was used to synthesize DNA fragments for targeted gene knockout of *fur* in *B. subtilis* (strain GP879, see appendix, chapter 9.5.2). For this purpose, 3 individual DNA fragments are required: first, a resistance cassette promoting resistance against chloramphenicol, erythromycin, kanamycin or spectinomycin was amplified from plasmids pDG646, pDG780, pDG1726 or pGEM-cat, respectively (Guerout-Fleury *et al.*, 1995). Second, about 1000 bp DNA fragments of the 5´ and 3´ flanking regions (here referred to as upstream and downstream fragment) of the targeted gene were amplified with *Phusion*™ DNA polymerase. The 3´end of the upstream fragment as well as the 5´end of the downstream fragment were extended into the target gene in a way that all expression signals of neighbouring genes remain intact, thus preventing polar effects. These three DNA fragments were joined and amplified in another PCR with *Extender*™ DNA polymerase: 150 ng of the up- and downstream fragment, respectively, and 300 ng of the resistance cassette were mixed, denaturated and cooled down briefly to allow joining of the fragments. Joining was possible by complementary sequences of 25 bp that were attached to the single fragments by the respective primers. Thus, the 3` end of the upstream fragment was linked with the 5` end of the resistance cassette and the 3`end of the resistance with the 5`end of the downstream fragment. In a second step the annealed fragments were elongated to obtain joined PCR products consisting of all three DNA fragments. The fused DNA fragments were then amplified in a third step by using the forward primer of the upstream and the reverse primer of the downstream fragment.

Long flanking homology PCR (LFH-PCR) with *Extender*™ DNA polymerase	
10x *Extender*™ buffer	10 µl
Upstream fragment	150 ng
Downstream fragment	150 ng
Resistance cassette	300 ng
Deionized H_2O	Ad. 87 µl
After step 1 addition of dNTPs and *Extender*™ DNA polymerase:	
dNTPs 12.5 mM	4 µl
Extender™ DNA polymerase	1 µl (2.5 U)
After step 2 addition of primers:	
Forward primer upstream fragment (20 pmol)	4 µl
Forward primer downstream fragment (20 pmol)	4 µl

LFH-PCR standard program for *Extender*™ DNA polymerase				
Step 1	Initial denaturation	94°C	2 min	
	Annealing	Stepwise to 15°C	5°C/min	
	Pause	15°C	∞	
Add dNTPs and *Extender*™ DNA polymerase, then proceed:				
Step 2	Elongation	68°C	8 min	
	Denaturation	94°C	20 sec	**10x**
	Annealing	As calculated	45 sec	
	Elongation	68°C	8 min	
	Pause	15°C	∞	
Add primers, then proceed:				
Step 3	Denaturation	94°C	20 sec	**30x**
	Annealing	As calculated	45 sec	
	Elongation	68°C	10 min	
	Hold	15°C	∞	

Combined chain reaction (CCR)

The combined chain reaction was used for site directed mutagenesis of *M. pneumoniae* promoters and UGA stop codons to allow recombinant expression of *M. pneumoniae* genes in

E. coli and *B. subtilis*. The principle is based on a PCR that contains an additional, third primer that inserts one or multiple point mutations during amplification. This mutagenic primer is characterized by its +10°C higher melting temperature *Tm* compared to the amplification primers and by its 5´ phosphorylation. The phosphorylation allows ligation to the 3´-OH group of the extended upstream primer by a thermo-stable DNA ligase. A high number of mismatching nucleotides in the mutagenesis primer decrease the efficiency of the reaction. Furthermore, these mismatches have to be located in the middle of the mutagenic primer. Therefore, if multiple mutations should be introduced during amplification, additional mutagenic primers are required. This allows up to seven point mutations in one step, a variation of the CCR named multiple mutation reaction (MMR) (Hames *et al.*, 2005). The CCR reaction was perfomed with 2.5 U of Accuzyme™ DNA polymerase (Bioline, Luckenwalde, Germany) and Ampligase® (Epicentre, Madison, USA) in CCR buffer (20 mM Tris-HCl pH 8.5, 3 mM $MgCl_2$, 50 mM KCl, 0.5 mM NAD^+) and 0.4 mg/ml bovine serum albumin (BSA, New England BioLabs, Ipswich, USA) in a total volume of 50 µl. To reduce unspecific amplification, a plasmid containing the native gene was used as a template instead of chromosomal DNA.

Combined chain reaction (CCR) with *Accuzyme*™ DNA polymerase (50 µl)	
Plasmid DNA	200 ng
10 x CCR buffer	5 µl
Forward primer (20 pmol)	2 µl
Reverse primer (20 pmol)	2 µl
Mutagenic primer (20 pmol)	4 µl
dNTPs 12.5 mM	1 µl
Bovine serum albumine (10 mg/ml)	2 µl
Ampligase®	3 µl (15 U)
Accuzyme™ DNA polymerase	1 µl (2.5 U)
Deionized H_2O	Ad. 50 µl

CCR standard program for *Accuzyme*™ DNA polymerase			
Initial denaturation	95°C	5 min	
Denaturation	95°C	30 sec	**30x**
Annealing	As calculated	30 sec	
Elongation	65°C	3 min/kb	
Final elongation	65°C	5 min	
Hold	15°C	∞	

Enzymatic digestion of DNA

The digestion of DNA with endonucleases was performed with buffers as recommended by the manufacturer. Reaction buffers, concentration of enzymes and DNA as well as incubation temperatures were chosen according to the manufacturer's instructions. The digestion was allowed to proceed for up to 2 h and was, if possible, followed by heat inactivation of the restriction endonucleases (20 min; 65°C or 80°C). The DNA was purified using the PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Dephosphorylation of 5´ends of DNA

To avoid re-circularization of a previously digested DNA vector, the 5' phosphate groups of the linearized vector were removed prior to the ligation reaction. The dephosphorylation of the 5`-prime end of DNA fragments was performed with the FastAP (alkaline phosphatase) (Fermentas, Lithuania) with buffers supplied by the manufacturer. Approximately 3-10 ng/µl DNA were mixed with 1 µl FastAP (1U/µl) and incubated at 37°C for 30 min. The FastAP was inactivated by incubation at 75°C for 10 min.

Ligation of DNA

DNA fragments were ligated using T4 DNA ligase (Fermentas, Lithuania) with buffers supplied by the manufacturer. The ligation reaction contained 20 - 200 ng of vector DNA and an excess of the DNA fragment (insert to vector molar ratio of 10:1 to 20:1). The reaction was started after addition of 5 U T4 DNA ligase in a final volume of 20 µl. The ligation occurred for 2 h at room temperature or overnight at 16°C.

Buffers for experiments with DNA

Lysis buffer (2.5 ml)	Lysozyme	50 mg
	Tris-HCl pH 8.0 (1 M)	50 µl
	EDTA pH 8.0 (0.5 M)	10 µl
	Ad H_2O 2.5 ml, make aliquots and store at -20°C.	
10x PBS (1 l)	KCl	2 g
	KH_2PO_4	2 g
	Na_2HPO_4 x2 H_2O	14.24 g
	NaCl	80 g
	pH 7. 4	
50x TAE buffer (500 ml)	Tris	242 g
	Acetic acid (100%)	57.1 ml
	EDTA pH 8.0 (0.5 M)	100 ml
	Deionized H_2O	ad 500 ml
5x DNA loading dye	Glycerol	5 ml
(10 ml)	TAE buffer (50x)	200 µl
	Deionized H_2O	4.5 ml
	Bromphenol blue	0.01 g
	Xylencyanol	0.01 g
Depurination buffer	250 mM HCl	
(Southern blot)		
Denaturation buffer (1 l)	NaCl	87.66 g
(Southern blot)	NaOH	20 g
Neutralisation buffer (1 l)	Tris HCl	121.14 g
(Southern blot)	NaCl	87.66 g
	pH 7.5	

20x SSPE buffer (1 l)	NaCl	175.3 g
	NaH_2PO_4 xH_2O	26.6 g
	EDTA	7.4 g
	pH 7.4	
20x SSC buffer (1 l)	Na_3-Citrate x2 H_2O	88 g
	NaCl	175.3 g
	pH 7.0	
	Autoclaving required.	
Pre-hybridisation buffer (400 ml)	Formamide	200 ml
	20x SSC	100 ml
	N-Lauroylsarcosin (10%)	4 ml
	Blocking reagent	8 g
	SDS	28 g
	Ad H_2O 400 ml, solve at 60°C and store at -20°C.	
Buffer 1 (Southern blot)	2x SSC buffer with 0.1% SDS	
Buffer 2 (Southern blot)	0.1x SSC buffer + 0.1% SDS	
5x Buffer DIG-P1 (1 l)	Maleic acid	58.04 g
	NaCl	43.83 g
	pH 7.5	
Buffer DIG-P2	Buffer DIG-P1 with 1% blocking reagent	
Buffer DIG-P3 (1 l)	Tris	12.1 g
	NaCl	5.8 g
	pH 9.5	

2.2.4 Experiments with RNA

All materials, buffers and consumables used to work with RNA were RNase free. Surfaces, pipettes and gloves were cleaned with 70% ethanol and 0.5% SDS prior to work. Unless mentioned otherwise, RNA was solved in RNase free water supplied from commercial systems or with DEPC treated water and stored at -80°C. RNA concentrations were measured with a ND-1000 NanoDrop (Peqlab, Erlangen, Germany).

DEPC treated water

Diethylpyrocarbonate (DEPC) is a chemical agent commonly used to inactivate RNases. DEPC is solved in water to a final concentration of 0.1%, stirred at room temperature for several hours and then autoclaved. RNases are inactivated by covalent modification of histidin residues, and unused DEPC resolves into ethanol, H_2O and CO_2 during autoclave treatment.

RNA preparation

RNA preparations for Slot blot and Northern blot analysis were prepared with the RNeasy® Midi Kit (Qiagen, Hilden, Germany). *M. pneumoniae* cultures (150 cm^2) were grown for 96 h at 37°C, adherent cells washed twice with PBS and lysed directly with 2 ml buffer RLT with 1% β-mercaptoethanol from the kit. The lysate was stored at -80°C or used for RNA preparations according to the manufacturer´s instructions.

If RNA was used for cDNA synthesis and microarray analysis, RNA preparation was performed with the RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) as described before (Güell *et al.*, 2009). *M. pneumoniae* cultures (150 cm^2) were grown for 6 h, 24 h, 48 h, 72 h and 96 h, adherent cells washed twice with PBS and then lysed directly by the addition of 1.5 ml buffer RLT plus β-mercaptoethanol. The lysate was stored at -20°C or used for RNA preparations according to the manufacturer´s instructions with the following modifications: two DNA Eliminator columns and two RNeasy spin columns per sample were used for cultures that had been grown for 6 h and 24 h. For samples that had been grown longer than 24 h, four DNA Eliminator columns and 4 RNeasy spin columns per sample were used. RNA was eluted in 50 µl RNase free water per column. The collected sample was dried in a SpeedVac at room temperature, the RNA pellet dissolved in 20 µl RNase free H_2O and stored at -80°C.

RNA gel electrophoresis

RNA was separated by electrophoresis for quality control and for Northern blotting. 1 – 5 µg RNA (10 µl final volume) were mixed with 5 µl RNA loading buffer (with ethidium bromide) and denaturated at 65°C for 10 min. The sample was cooled on ice, briefly centrifuged and loaded on a 1.5% agarose gel containing 6% formaldehyde. The gel ran for 120 min at 100-120 V in MOPS buffer. Visualisation and documentation were performed with a UV transilluminator 2000 (Bio-Rad, Munich, Germany) by exposing the gels to UV light (λ = 254 nm).

Bioanalyser

The quality of RNA used for cDNA synthesis and microarray analysis was randomly controlled with a 2100 Bioanalyzer (Agilent).

Slot blot /Northern blot analysis

Slot blots are a variant of dot blots in which RNA is directly transferred onto a nylon membrane without separation by gel electrophoresis. In contrast to dot blots, RNA is transferred by vacuum through a small slot, thereby forcing it onto a small, defined space on the membrane. As for dot blots, a defined amount of whole RNA (10 µg) is diluted in 20x buffer and a dilution series in steps of 10^{-1} are prepared. These dilution series are blotted onto the membrane and can be analyzed like Northern blots.

For Northern blotting, RNA was first separated by agarose gel electrophoresis and then transferred onto a nylon membrane as described for Southern blotting with the following changes: No depurination buffer was used, and incubation with denaturation buffer and neutralisation buffer was only done for 5 min. 20x SSC buffer was used for the transfer instead of 20x SSPE buffer

Visualisation and analysis of Northern blots and Slot blots with DIG labelled RNA probes was performed as described for Southern blots.

Microarray analysis

Microarray analysis was performed as described before (Güell *et al*., 2009). Cultivation of strains, RNA preparation, cDNA synthesis and Cy5 labelling of cDNA as well as data evaluation were carried out at the CRG, Barcelona, Spain, with assistance of Eva Yus. Hybridisation, scanning of microarrays and data processing were performed by Sabine

Schmidt at the Genomics Core Facility, EMBL, Heidelberg, Germany. Marc Güell (CRG, Barcelona) helped with the data analysis.

cDNA synthesis and Cy5 labelling were conducted with the SuperScript™ Indirect cDNA Labeling System (Invitrogen), the S.N.A.P.™ Column Purification module (Invitrogen) and the Cy5™ Mono-Reactive Dye module (Amersham Biosciences) in accordance to the manufacturer´s instructions with the following modifications: 9 µg of total RNA were mixed with 0.84 µl random hexamer primers and water to a final volume of 18 µl. For annealing of hexamer primers to RNA the samples were incubated at 70°C for 5 min and then chilled on ice for at least one minute. A master mix was prepared consisting of 6 µl 5x First-Strand buffer, 1.5 µl DTT, 1.5 µl modified dNTP mix, 1 µl RNaseOUT™ (40 U/µl) and 2 µl SuperScript™ III RT (400 U/µl) per sample. 12 µl of the master mix were added to each sample and mixed gently. Samples were incubated at 37°C for 3 – 4 h. cDNA concentration was measured with a ND-1000 NanoDrop (Peqlab, Erlangen, Germany).

Design and manufacture of the microarray as well as hybridisation conditions have been described before (Güell *et al.*, 2009). All experiments were performed as one-channel experiments (one sample per microarray, no competitive hybridisation of two different labelled samples).

Microarray data was processed with software *R* (www.r-project.org) and *Excel* (Microsoft). For statistical analysis, quantile normalization was done using the bioconductor package *marray* after background substraction (www.bioconductor.org). Differential expression analysis of array data was performed using *R limma* package.

Synthesis of DIG labelled RNA probes by *in vitro* transcription

Digoxygenin (DIG) labelled RNA probes were used to detect and quantify nucleic acids in Southern, Northern and slot blot experiments. The probes are *in vitro* transcribed *anti-sense* RNAs of 500 – 1000 bp length that consist of labelled nucleotides which can be recognized by specific antibodies.

A DNA fragment of the intragenic region of the gene of interest was amplified by Phusion™ PCR. The fragment contained a T7 RNA polymerase promoter at the 5´end of the non-coding strand that was added by the reverse primer:

5´-CTAATACGACTCACTATAGGGAGA-primer-3´

The DNA fragment was purified with the QIAquick PCR Purification Kit (Qiagen) and used as a template for *in vitro* transcription by T7 RNA polymerase. The following reaction was prepared:

***In vitro* synthesis of DIG labelled RNA probes (20 µl reaction)**	
Template DNA	13 µl
10x T7 transcription buffer	2 µl
DIG RNA labelling mix	2 µl
RNase Inhibitor	1 µl
T7 RNA polymerase	2 µl

T7 transcription buffer, T7 polymerase, DIG RNA labelling mix and RNase Inhibitor were purchased from Roche Diagnostics. The reaction was incubated at 37°C for up to 4 h and then stopped by the addition of 1 µl 0.5 M EDTA (pH 8.0). RNA was precipitated by the addition of 2.5 µl 4 M LiCl and 75 µl ice-cold ethanol and by overnight incubation at -80°C. RNA was sedimented by centrifugation (30 min, 13000 rpm and 4°C), the pellet washed with 70% ice-cold ethanol and then dried at room temperature to remove traces of ethanol. The RNA was eluted in 100 µl DEPC treated water containing 1 µl RNase inhibitor (Roche Diagnostics) and stored at -80°C. To control the quality of the RNA probe, dilution series (in steps of 10^{-1}) were prepared, 1 µl of each dilution step spotted onto a nylon membrane and luminescence tested as described previously (see Southern Blots).

Buffers for experiments with RNA

RNA loading buffer	Formamide	6.5 ml
(10 ml)	Formaldehyde	1.2 ml
	10x MOPS buffer	2 ml
	Sucrose (50%)	0.4 ml
	Bromphenol blue	10 mg
	Xylencyanol	10 mg

10x MOPS buffer	MOPS	41.8 g
(1 l)	Sodium acetate	6.8 g
	EDTA (0.5 M)	20 ml
	H_2O	ad 1000 ml
	pH 7.4	
	Do not autoclave. Store at 4°C in the dark.	

2.2.5 Experiments with proteins

Unless mentioned otherwise, proteins were stored for short terms in aqueous solution at 4°C or for long terms in buffers containing 40% glycerol and DTT at -20°C.

Overexpression of recombinant proteins in *E. coli*

Overexpression from plasmids based on pWH844 was performed in *E. coli* DH5α, and overexpression from plasmids based on pGP172 (T7 RNA polymerase dependent expression) was performed in *E. coli* BL21. An overnight culture of *E. coli,* carrying the relevant plasmid, was used to inoculate one litre of LB medium to OD_{600} ~ 0.1. Cultures were grown with shaking at 16°C or 37°C until they had reached an optical density of 0.6 - 0.8. Expression of recombinant proteins was induced by the addition of isopropyl-β-D-thio-galactopyranoside (IPTG, final concentration: 1 mM) (PeqLab, Erlangen, Germany). The cultures were incubated for three more hours. To test the expression, small aliquots (sample [µl] = $100/OD_{600}$) were taken before and after the induction. The samples were boiled in SDS loading dye and analyzed on a SDS gel. The main culture was harvested by centrifugation (10 min; 8,000 rpm; 4°C). After removing the supernatant the cells were washed in cold cell disruption buffer and the pellets were stored at -20°C.

Disruption of bacterial cells

E. coli and *B. subtilis* cells were disrupted using a French press or a One shot device. *M. pneumoniae* was disrupted by sonication or using a One shot device.

French press

The prechilled bomb was filled with the cell suspension and the remaining air was squeezed out before the bomb was locked. After closing the release valve the bomb was placed in the French press and set under pressure. The disruption took place with a pressure of 1400 bar and was performed three times.

One Shot

The cell disruption with the One Shot was in principle similar to the French press. Here, 6 ml of the cell suspension were pipetted into the precooled cylinder and closed airtight. Internal pressure was established using the starter stick. The disruption of the cells was carried out at maximum pressure (2,500 psi; 2 to 3 passages)

Sonication

Harvested cells were suspended in PBS buffer and placed on ice. Sonication was performed 5 – 10 times 20 sec (60 W, 0.5 sec clearance).

Protein purification

Proteins were purified from crude extracts by affinity purification. Overexpressed proteins carry a His_6 or STREP affinity tag that allows binding to and controlled elution from a relevant matrix.

Purification of His_6 tagged proteins

The frozen pellets were resuspended in cold ZAP buffer (200 mM NaCl, 10 mM Tris-HCl pH 7.5), and the cells were disrupted using the French pressure cell. Cell debris and other insoluble material were removed by ultracentrifugation (Beckmann, L7-55) (60 min; 27.000 rpm; 4°C). For purification of recombinant His_6-tagged proteins the supernatant fraction was loaded onto a 4 ml bed volume of Ni^{2+}-NTA resin (Qiagen) in a Poly-Prep Chromatography Column (Biorad, Munich, Germany). The Ni^{2+}-NTA resin had been pre-equilibrated with 10 ml ZAP buffer. After extensive washing with 20 ml of ZAP buffer containing 10 mM imidazole (Roth, Karlsruhe, Germany) and 20 ml with 20 mM imidazole the His_6-tagged proteins were eluted. The elution was performed in 10 ml steps with ZAP buffer containing an increasing concentration of imidazole (50 mM, 100 mM, 200 mM, and 500 mM). To analyze the purification success, 15 µl of each fraction was mixed with SDS loading dye and boiled at 95°C for 10 minutes. The samples were loaded onto a SDS gel and after electrophoresis stained with Coomassie brilliant blue. The relevant fractions were combined and dialyzed overnight. Protein concentration was determined using the Bio-Rad dye-binding.

Purification of STREP tagged proteins

Overexpression and cell disruption were performed as described above. The proteins were purified using 1 ml Strep-Tactin Sepharose (IBA, Göttingen, Germany) loaded on a Poly-Prep

Chromatography Column (Biorad, Munich, Germany). The matrix specifically binds a sequence of eight amino acids (WSHPQFEK). D-desthiobiotin was used to elute the bound proteins. The matrix was equilibrated with 5 ml of buffer W. Afterwards the column was loaded with 10 ml of the crude extract. Washing steps were performed with 5 ml buffer W. The bound proteins were eluted with buffer E in 6 fractions of 500 µl. The fractions were analyzed by SDS-PAGE

Dialysis of purified proteins

Fractions containing eluted protein from Ni^{2+}-NTA or STREP purifications contain high concentrations of imidazole or D-desthiobiotin, respectively, which both can interfere with further applications. To remove imidazole or D-desthiobiotin, elution samples were dialyzed in a dialysis tube (3.5 kDa exclusion size, boiled in 100 mM EDTA for 10 min) against protein storage buffer (in a ratio 1:2000) for 24 h at 10°C.

Protein concentration measurements

Protein concentration was determined either with the Bio-Rad dye-binding assay (Bio-Rad) or the BCA Protein assay (Pierce) using bovine serum albumin as the standard.

Discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970). Protein samples were mixed with SDS loading dye and heated at 95°C for 10 min to denature before loading onto the gel. The polyacrylamide (PAA) concentration of the gels was chosen according to the expected proteins sizes, varying from 10 – 16% (v/v). Gels were run at 120 V in electrophoresis buffer at room temperature until the bromphenol blue had reached the lower end of the gel. Gels consist of two phases with different PAA concentrations: a thin, low concentrated stacking phase in which proteins are focussed, and a running gel of 10 – 16% PAA concentration in which proteins are separated during electrophoresis according to their molecular mass. Self-constructed unstained protein marker (*Page King*) or pre-stained, purchased protein markers (Fermentas, Lithuania) were used as size standards. Gels were either used for Western blotting or analyzed by Coomassie or silver staining.

Coomassie staining of polyacrylamide gels

Coomassie staining was most commonly used to analyse protein gels. Gels were incubated in fixing solution for 5 – 10 minutes, stained with Coomassie solution for up to 15 minutes and

destained until the contrast between protein bands and background was optimal. For documentation, the gels were put in plastic wrap and scanned.

Silver staining of polyacrylamide gels

Silver staining of protein bands was performed as described by Nesterenko (1994). Silver staining is highly sensitive and has a detection limit of approximately 5 ng protein. In contrast to Coomassie staining it is more time consuming, less reproducible and not quantifiable, due to the physics of silver accumulation in the gel (Butcher and Tomkins, 1985). For staining, polyacrylamide gels were incubated in the following solutions at room temperature with shaking:

Step	Reagent	Duration
Fixing	Fixing solution	1 to 24 h
Washing	Ethanol 50%	3x 20 min
Reduction	Thiosulfate solution	90 sec
Washing	Deionized H_2O	3x 20 sec
Staining	Impregnating solution	25 min
Washing	Deionized H_2O	2x 20 sec
Developing	Developer	Until sufficiently stained
Washing	Deionized H_2O	5 sec
Stopping	Stop solution	5 min

Western blot analysis

Western blots (Towbin *et al.*, 1979) allowed the detection and quantification of STREP tagged proteins after SDS-PAGE by using an anti-$GlpK_{Mpn}$ antibody (Hames *et al.*, 2005). Proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) with a semi-dry blotting apparatus (Amersham). The PVDF membrane was activated in methanol and equilibrated in transfer buffer. A stack of following layers was prepared (from bottom to top): 2 layers Whatman paper soaked with transfer buffer, activated and equilibrated PVDF membrane, PAA gel (unstained), 2 layers of Whatman paper soaked with transfer buffer. The stack was loaded on the blotting apparatus and proteins were transferred for 1 h at 0.8 mA/cm^2. Protein transfer to the membrane was controlled by staining of the membrane with 1 ml Ponceau S. The membrane was incubated in Blotto overnight at 10°C

while shaking to remove Ponceau S and to block unspecific binding sites on the membrane. Antibodies were diluted in Blotto and incubated with the membrane for 3 h at room temperature while shaking. The antibody was removed, the membrane washed three times 30 min with Blotto and then incubated with a secondary antibody (anti-rabbit IgG coupled with alkaline phoshatase from Promega, diluted 1:100000) for 30 min at room temperature. Again, the membrane was washed 3 times 30 min in Blotto, shortly rinsed with water and then equilibrated 5 min in buffer DIG-P3 (see chapter 2.2.3, Southern blot). Proteins were detected and quantified by chemiluminescence using CDP* as a substrate for alkaline phosphatase as described for Southern blots.

Protein identification by mass spectrometry

Protein bands were excised from the gel with a scalpel and analysed by mass spectrometry. This was performed by Dörte Becher (University Greifswald, Germany) or by the Zentrum für Molekulare Medizin, Zentrale Bioanalytik (ZBA, University of Cologne, Germany).

Buffers for experiments with proteins

ZAP buffer	NaCl	200 mM
	Tris-HCl pH7.5	50 mM
Buffer W	Tris-HCl pH 8.0	100 mM
	NaCl	150 mM
	EDTA	1 mM
Buffer E	Tris-HCl pH 8.0	100 mM
	NaCl	150 mM
	EDTA	1 mM
	D-desthiobiotin	2.5 mM

Protein storage buffer	Tris-HCl pH 7.9	20 mM
	EDTA	0.1 mM
	KCl	100 mM
	Glycerol	50%
	Tween 20	0.5%
	Autoclave, then add before usage:	
	DTT	1 mM
5x SDS loading dye	Tris-HCl pH 7.0 (1 M)	1.4 ml
(10 ml)	Glycerol	3 ml
	SDS (20%)	2 ml
	β-mercaptoethanol	1.6 ml
	Bromphenol blue	0.01 g
	Deionized H_2O	2 ml
6% Stacking gel (v/v)	Acrylamide-Bisacrylamide	740 µl
(SDS-PAGE)	(39:1, 40%)	
	Tris-HCl pH 6.8 (1 M)	650 µl
	SDS (20%)	50 µl
	APS (10%)	200 µl
	Deionized H_2O	5.35 ml
	TEMED	10 µl
12% Running gel (v/v)	Acrylamide-Bisacrylamide	4.6 ml
(SDS-PAGE)	(39:1, 40%)	
	Tris-HCl pH 8.8 (1 M)	1.3 ml
	SDS (20%)	200 µl
	APS (10%)	200 µl
	Deionized H_2O	6.5 ml
	TEMED	15 µl
10x Electrophoresis buffer	Glycine	1.92 M
(SDS-PAGE)	Tris	0.5 M
	SDS	10%

Fixing solution	Methanol	50%
(Coomassie staining)	Acetic acid	10%
	Deionized H_2O	40%
Staining solution	Coomassie Brilliant	2.5 g
(Coomassie staining)	Blue R250	
	Methanol	50%
	Acetic acid	10%
	Deionized H_2O	40%
	(Can be used several times!)	
Destaining solution	Acetic acid	10%
(Coomassie staining)	Deionized H_2O	90%
Fixing solution (100 ml)	Methanol	50 ml
(Silver staining)	Acetic acid	12 ml
	Formaldehyde (37%)	100 µl
	Deionized H_2O	Ad. 100 ml
Thiosulfate solution	$Na_2S_2O_3$ x5 H_2O	20 mg
(100 ml)	Deionized H_2O	Ad. 100 ml
Impregnating solution	$AgNO_3$	0.2 g
(100 ml)	Formaldehyde (37%)	37 µl
(Silver staining)	Deionized H_2O	Ad. 100 ml
Developer (100 ml)	Na_2CO_3	6 g
(Silver staining)	Thiosulfate solution	2 ml
	Formaldehyde (37%)	50 µl
	Deionized H_2O	Ad. 100 ml
Stop solution (100 ml)	EDTA	1.86 g
(Silver staining)	Deionized H_2O	Ad. 100 ml

Transfer buffer (5 l)	Tris	15.1 g
(Western Blot)	Glycine	72.1 g
	Methanol	750 ml
	Deionized H_2O	Ad. 5 l
10x TBS (1 l)	Tris	60 g
(Western Blot)	NaCl	90 g
	Deionized H_2O	Ad. 1 l
	pH 7.6	
Blotto (1 l)	10x TBS	100 ml
(Western Blot)	Skim milk powder	25 g
	Tween 20	1 ml
	Deionized H_2O	Ad. 1 l

2.3 Metabolite analysis by liquid chromatography-mass spectrometry (LC-MS)

LC-MS was used to monitor (p)ppGpp synthesis and to analyse the metabolome of *M. pneumoniae.* The author performed cultivation of bacterial strains and sample preparation, LC-MS analysis and data processing were performed by Manuel Liebeke at the University of Greifswald as described before (Geiger *et al.*, 2010).

For the detection of (p)ppGpp, *M. pneumoniae* strains were grown in Hayflick medium at 37°C. After 96 h growth, the medium was discarded and adherent cells were washed twice with PBS buffer pre-warmed to 37°C. Traces of buffer were removed and cells covered with 25 ml pre-warmed „Hayflick light“ (modified Hayflick´s medium with 1% glucose and 1 % glycerol, but without horse serum and phenol red) with or without 1mg/ml (8,3 mM) DL-serine hydroxamate (Sigma-Aldrich). After an incubation at 37°C for indicated time points, adherent cells were lysed with 10 ml ice cold 60% ethanol (HPLC grade, Sigma-Aldrich) and 100 µl 1 mM 8-bromoadenosine 5′-triphosphate (Sigma-Aldrich) which served as an internal standard. The cell lysate was transferred into a 15 ml Falcon tube and stored in liquid nitrogen until all samples were collected. All samples were than thawed while centrifuged (5000 rpm, 2°C), the supernatant transferred into a fresh 50 ml Falcon tube on ice and the remaining pellet supended in 10 ml HPLC water. Cell debris was pelleted again by centrifugation and the supernatant transferred into the 50 ml Falcon tube. This extraction step was repeated once. Samples were frozen in liquid nitrogen, stored at -80°C and shipped to Greifswald on dry ice.

2.4 Bacterial adenylate cyclase based two-hybrid system (BACTH)

The bacterial adenylate cylase based two-hybrid system (Karimova *et al.*, 1998; distributed by Euromedex, France) was used to study protein-protein interactions *in vivo* in heterologous *E. coli*. It is based on the interaction-mediated reconstitution of catalytic activity of the adenylate cyclase CyaA from *Bordatella pertussis* (Ladant and Ullmann, 1999). The catalytic domain of CyaA consists of two complementary fragments (T25 and T18) that loose enzymatic activity when physically separated. Activity can be restored when the two fragments are fused to interacting proteins, resulting in interaction of T25 and T18, and hence, in cAMP synthesis. cAMP binds to *E. coli* catabolite activator protein (CAP), thereby activating its function as a pleiotropic transcription regulator and allowing transcription of the reporter genes, such as *lacZ* . Since heterodimerization of the proteins fused to T25 and T18 is a prerequisite for reconstitution of CyaA activity, β-galactosidase activity can be used as a qualitative indicator for protein-protein interactions (see figure 2.2 below).

To detect protein-protein interactions, the T25 and T18 domains were either fused to the N- or the C-terminus of the target proteins. The plasmids used to construct these fusions can be found in the appendix (chapter 9.3.2) of this work. Genes of putatively interacting proteins were amplified by PCR and cloned into the vectors between restriction sites *XbaI* and *KpnI*, in frame to the respective sequence of the T25 or T18 domain. If necessary, opal codon UGA coding for tryptophane in *M. pneumoniae* but acting as a stop codon in *E. coli* was mutated by CCR (see chapter 2.2.3) to allow expression in *E. coli*. To prevent cytotoxic effects by the expression of fusion proteins and selection towards suppressor mutants, cloning was performed in *E. coli* XL1-Blue. This strain over-expresses the repressor *lacI*, thereby blocking transcription of fusion genes.

Co-expression of fusion proteins was performed in *E. coli* BTH101 (Δ*cya*). 5 ng of each pUT18/pUT18C and pKT25/p25-N derivative were mixed with 30 µl competent BTH101 cells and incubated on ice for 30 min. After a heat shock (42°C, 2 min), the cells were mixed with 120 µl LB medium and incubated at 30°C for 2 h. 4 µl of the sample were plated on LB medium containing ampicillin, kanamycin and X-gal and incubated at 30°C. Pictures were taken after 24 h, 36 h and 48 h. Protein interactions could be identified by the blue colour of the colonies.

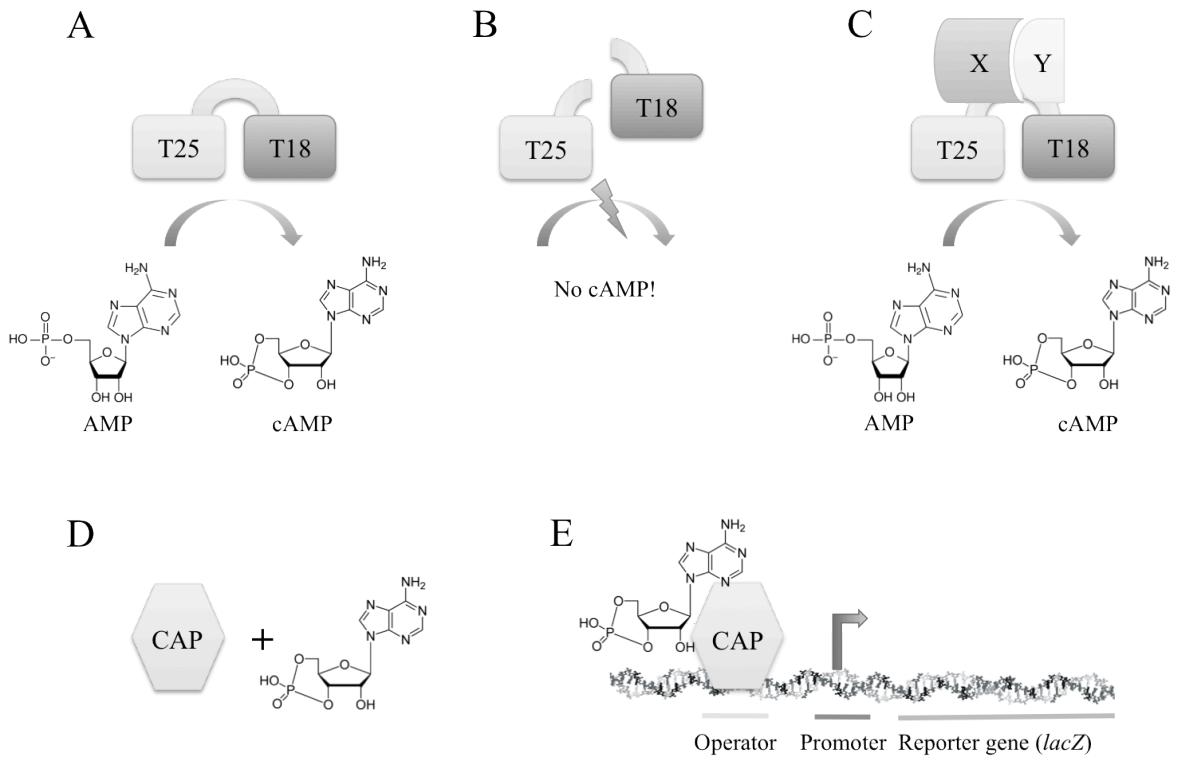


Figure 2.2: The bacterial-2-hybrid system (BACTH)

Physical contact of domains T25 and T18 is a prerequisite for enzymatic activity of CyaA (A, B). Physical contact can be mediated by artificial fusions of domains T25 and T18 to interacting proteins X and Y, thereby restoring adenylate cyclase activity (C). cAMP is a required cofactor of transcription regulator CAP in *E. coli*. In the presence of cAMP, CAP binds to operators of the *lacZ* reporter gene, thereby inducing transcription (D, E). Hence, β-galactosidase activity directly correlates with the interaction of proteins X and Y. Figure taken and modified from the BACTH manual.

2.5 Chromatin affinity purification (ChAP): SPICE

Chromatin affinity precipitation (ChAP) is a variation of widely used chromatin immunoprecipitation (ChIP). In ChIP, DNA binding proteins are cross-linked to specifically bound DNA *in vivo,* and protein-DNA complexes then purified by immunoprecipitation using specific antibodies against the target protein. In ChAP, protein-DNA complexes are affinity purified by a protein tag like His_6, TAP or STREP (Ishikawa *et al.*, 2007). Cross-linked, co-purified DNA can be recovered and analysed by qPCR, sequencing or microarray analysis (ChAP-chip). In this work, a modified ChAP protocol was developed as an attempt to identify the DNA binding-sites of putative transcription regulators in *M. pneumoniae*.

First, plasmids to over-express putative transcription regulators with a C-terminal STREP tag in *M. pneumoniae* were constructed based on plasmid pMT*clpB*-TAP (Kühner *et al*., 2009).

M. pneumoniae was transformed with these plasmids, spread on Hayflick agar plates containing gentamycin and single colonies picked to grow homogeneous insertion strains. Expression of STREP-tagged proteins was controlled by Western blotting using anti-$GlpK_{Mpn}$ antibodies that do also recognize STREP-tagged proteins (Hames *et al.*, 2005). For ChAP, two liters of culture (10 culture flasks of 300 cm^2) were grown in Hayflick medium with glucose and gentamycin for 96 h. Cross-linking was either performed by covering adherent cells with 0.2% para-formaldehyde (solved in PBS, pH 6.5) for 10 min at 30°C after washing, or by the addition of 0.2% (final concentration) para-formaldehyde to cells harvested in PBS and 10 min incubation at 30°C while shaking (90 rpm). Cross-linking was stopped by centrifugation of the cells and washing the pellet once with PBS. The pellets were stored at -80°C until further used.

The pellet was suspended in 15 ml buffer W (see chapter 2.2.5) and cells were disrupted using the OneShot manifold. DNA was sheared by sonication and debris removed by centrifugation (8500 rpm, 4°C, 15 min). STREP-tagged proteins were purified with 500 µl Streptactin sepharose, purifcation controlled by SDS-PAGE and Western blotting and cross-linking reversed by overnight incubation of at 67°C. DNA was recovered from samples using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), DNA concentration determined with a ND-1000 Nanodrop photospectometer (Peqlab), and analyzed by qPCR (see chapter 2.2.3).

2.6 Characterisation of *M. pneumoniae* mutants

In this chapter, some experiments to characterise *M. pneumoniae* mutants are described.

Growth curves

Growth curves of *M. pneumoniae* strains were performed by measuring the wet weight of cultures as described previously (Halbedel *et al.*, 2004). At indicated time points, the medium was discarded, cells harvested in 1.5 ml PBS and transferred to an Eppendorf cup. Cells were sedimented by centrifugation (5 min, 13000 rpm), all liquid removed with a pipette and the weight determined with a balance. The wet weight is calculated by subtracting the weight of the empty Eppendorf tube from the weight of the Eppendorf tube with *M. pneumoniae* cells.

HeLa/A549 cytotoxicity assay

HeLa and A549 cell cytotoxicity assays were performed as described previously (Hames *et al.*, 2009). HeLa and A549 cell lines were grown in 24-well plates at 2.5 x10^4 cells per well in 700 µl Dulbecco modified Eagle medium (DMEM) for 24 h at 37°C with 5% CO_2. *M. pneumoniae* cultures were grown for 96 h at 37°C. *M. pneumoniae* cells were washed three times with 67.6 mM HEPES (pH 7.3), 140 mM NaCl, and 7 mM $MgCl_2$. The cells were scraped with 1 ml buffer and resuspended with a 0.4 by 20-mm syringe. Depending on the size of the pellet, it was resuspended in 5 to 8 ml buffer. HeLa/A549 cells were infected with an MOI (multiplicity of infection) of 1 x10^6: 0.1 A_{550} units were taken from the suspension and centrifuged for 5 min at 10000 rpm at 4°C. The pellet was resuspended in 125 µl of modified Hayflick medium with a 0.4x20 mm needle. The cells were then pipetted onto the lawn of HeLa or A549 cells and incubated for 2 h at 37°C and 5% CO_2. Then the supernatant was removed and replaced by 700 µl fresh Dulbecco modified Eagle medium.

Cell cultures were monitored for 120 h. To investigate the cytotoxicity of *M. pneumoniae* strains towards HeLa/A549 cells, the medium was removed from one micro titre plate well at each time point. The cells were incubated with 100 µl methyl violet solution (0.75% methyl violet, 0.25% NaCl, 1.75% formaldehyde and 50% ethanol) for 10 min at room temperature. After this, cells were washed twice with 600 µl of water, dried and pictures were taken. Cytotoxicity was judged by HeLa/A549 cell lysis in samples infected with *M. pneumoniae*.

Determination of *in vivo* hydrogen peroxide production

The hydrogen peroxide production by *M. pneumoniae* was determined as described previously (Hames *et al.*, 2009) The Merckoquant peroxide test (Merck, Darmstadt, Germany), which has a detection range of 0.5 to 25 µg of hydrogen peroxide per ml of solution, was used. The supernatant of a 100 ml culture was discarded, and the cells were washed twice with a buffer containing 67.6 mM HEPES (pH 7.3), 140 mM NaCl, and 7 mM $MgCl_2$. The cells were scraped with 1.5 ml buffer, transferred into a fresh tube, and centrifuged (10 min, 10000 rpm, 4°C). The pellet was washed with 1 ml and finally resuspended in 4 ml of the same buffer. Aliquots of 1 ml were adjusted to an A_{550} of 1.0. After incubation for 1 h at 37°C, glycerol or glucose (final concentration, 100 mM) was added to one aliquot. An aliquot without any added carbon source served as the control. The test strips were dipped into the suspensions for 1 sec and subsequently read.

Scanning electron microscopy

Maria Lluch-Senar performed scanning electron microscopy experiments of *M. pneumoniae* strains at the Universitat Autònoma de Barcelona (Spain).

After growing to mid-log phase in 5 ml of Hayflick medium, cells were scraped off and passed ten times through a syringe (0.2 mm diameter). Then, 20 µl of this cell suspension were inoculated to 2 ml of modified Hayflick medium containing glucose in a Lab-Tek chamber slide (Nunc). After growing cells to mid-log phase, the medium was removed and the cells were washed three times with PBS and fixed with 1% glutaraldehyde for 1h. Samples were washed three times with PBS and then dehydrated sequentially with 30, 50, 70, 90 and 100% ethanol for 10 min each. Samples were point dried immediately (K850 critical point drier; Emitech Ashfort, United Kingdom) and subsequently coated with 20 nm of gold. Samples were observed using a Hitachi S-570 (Tokyo, Japan) scanning electron microscope.

3. Results

3.1 Transposon insertions in genes putatively involved in transcription regulation

The most important tools to characterize the function of genes *in vivo* are genetic knockouts. Former studies aimed to characterize essential genes in *M. pneumoniae* and its relative, *M. genitalium* by random transposon mutagenesis (Hutchison *et al.*, 1999; Glass *et al.*, 2006). However, these studies were shown to comprise errors due to the experimental approach that did not successfully differentiate between viable and non-viable transposon insertions. For example, the *ldh* gene coding for lactate dehydrogenase was found to be essential in Hutchison *et al.*, 1999, whereas a viable *ldh* mutant was isolated in later works (Halbedel *et al.*, 2007). On the other hand, a transposon insertion in open reading frame Mpn352 coding for the only sigma factor *sigA* was found in Hutchison *et al.*, 1999. This observation is rather unlikely since this protein is essential to initiate transcription and could not be confirmed by this work. Therefore, to date, screening of the transposon library described previously (Halbedel *et al.*, 2006) is the most reliable method to determine essentiality of genes and the only method to isolate desired transposon insertion mutants of target genes in *M. pneumoniae*.

3.1.1 Screening for *M. pneumoniae* transposon insertion mutants

The *M. pneumoniae* transposon library was screened for transposon insertions in genes putatively involved in transcription and transcription regulation as described previously (Halbedel *et al.*, 2006). With the exception of Mpn397/RelA and Mpn241/WhiA, all tested genes were found to be essential in this study. Mpn244/DisA, Mpn273/Hit and Mpn329/Fur were reported to be not essential in previous studies but could not be isolated from the transposon library. Therefore, they are thought to be essential for viability. As expected, no disruption mutant of Mpn352/SigA could be isolated. In addition, the essentiality of Mpn266/Spx and Mpn626/SigD-like could be confirmed.

An overview about the essentiality of genes presumably involved in transcription or transcription regulation is given in table 3.1. The essentiality of the transcription regulators Mpn239/GntR and Mpn124/HrcA has been shown before (S. Halbedel, personal communication), and mutants Mpn223/HPrK and Mpn247/PrpC have been isolated and described previously (Halbedel *et al.*, 2006). The primers used in this study to screen for transposon insertion mutants can be found in the appendix (chapter 7.2.2) of this work.

Orf/ Description	Screening primer	Essential in previous studies	Essential in this study/strain designation	Author
mpn266/*spx*-like	HE272/HE273	Yes	Yes	This work
mpn241/*whiA* family	HE324/HE325	No#	No/GPM22	This work
mpn244/disA family	HE326/HE327	No#	Yes	This work
mpn273/*hit*-like	HE328/HE329	No#	Yes	This work
mpn329/*fur* family	HE181/HE182	No#	Yes	This work
mpn352/sigA	HE238/HE239	No#	Yes	This work
mpn397/relA	HE285/HE286	No#	No/GPM21	This work
mpn626/sigD-like	HE179/HE180	Yes	Yes	This work
mpn124/hrcA	SH91/SH92	Yes	Yes	S. Halbedel, 2006
mpn239/gntR family	SH87/SH88	Yes	Yes	S. Halbedel, 2006
mpn223/hprK	KS9/KS10	No#	No/GPM51	Halbedel *et al.*, 2006
mpn247/prpC	SH67/SH29	Yes	No/GPM68	Halbedel *et al.*, 2006

Tab. 3.1: Essentiality of genes in *M. pneumoniae* putatively involved in transcription regulation.
#, not essential in either Hutchison *et al.*, 1999 or Glass *et al.*, 2006. The primers used to screen for these mutants are either found in the appendix of this work or of the corresponding publications.

3.1.2 Verification of *M. pneumoniae* transposon insertion mutants

M. pneumoniae strains with putative transposon insertions in *mpn241/whiA* and *mpn397/relA* were isolated from the transposon library and further analyzed to determine the transposon insertion site and the number of transposon insertions. Genes were regarded to be inactivated if the transposon insertion fulfilled the Glass criteria established previously (Glass *et al.*, 2006).

mpn397/relA

Three *M. pneumoniae* strains with transposon insertions in *mpn397/relA* were isolated from the library. The PCR fragments obtained from these strains using primer pairs HE285/SH30 and HE286/SH29 were sequenced and aligned with the MPN397/RelA DNA sequence to identify the transposon insertion sites. All three transposon insertions met the Glass criteria. Two strains, namely F7.2-2 and F5.29-1, have transposon insertions between bases 546/547 and 769/770 in respect to the first base of the gene, respectively. Clone A7.31-2 harbours a transposon insertion between bases 904/905, thereby disrupting the gene within the region coding for the putative (p)ppGpp synthesis domain described previously (Sajish *et al.*, 2007). Therefore, this clone was chosen for further studies and it was renamed GPM21.

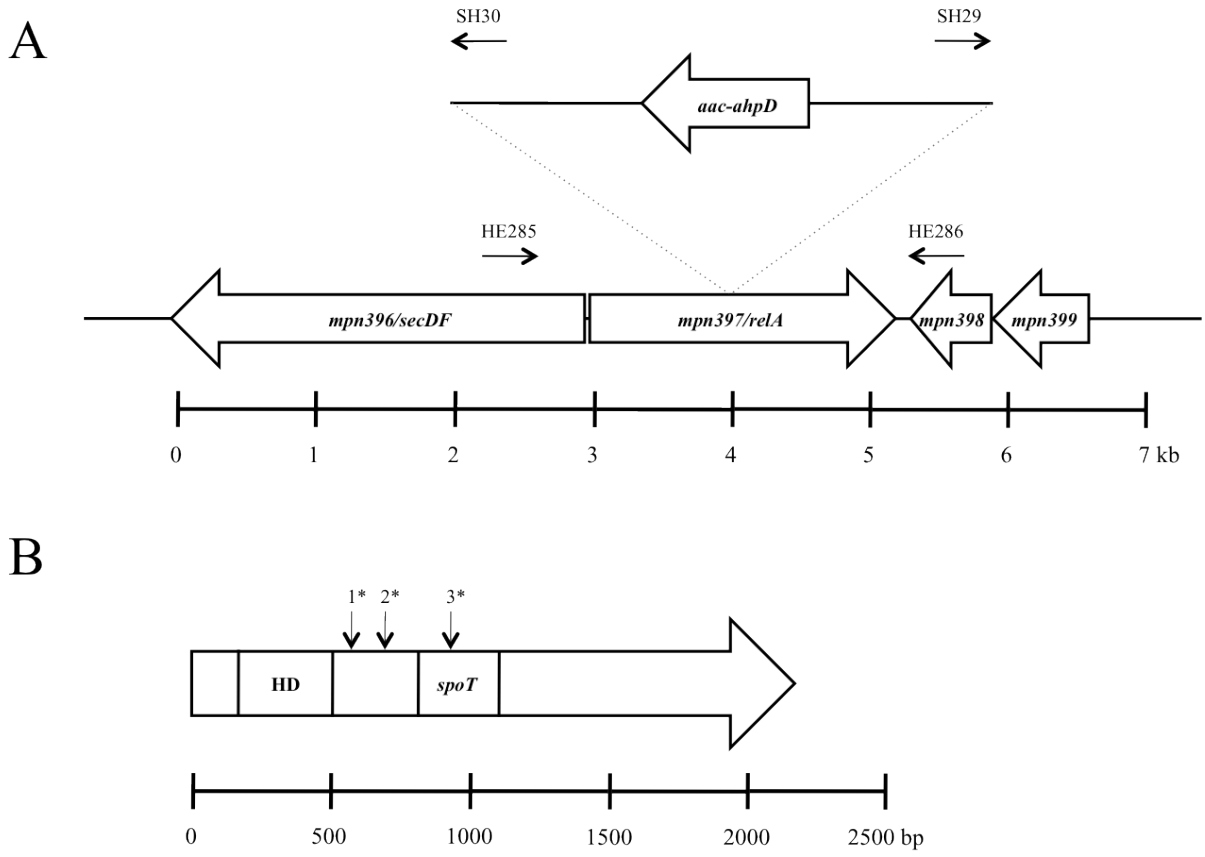


Fig. 3.1: Isolation of *mpn397* transposon insertion mutants.

A: Schematic overview of the screen and orientation of the transposon insertion; B: Schematic overview of the three different transposon insertions in clones F7.2-2 (1*), F5.29-1 (2*) and A7.31-2 (3*) in respect to the sequences coding for the conserved (p)ppGpp hydrolysis (HD) and (p)ppGpp synthesis (*spoT*) domains (Aravind and Koonin, 1998; Sajish *et al.*, 2007). F7.2-2 (1*), F5.29-1 (2*) and A7.31-2 (3*) harbour transposon insertions after bases 546, 769 and 904 in respect to the first nucleotide of the gene, respectively.

Strain GPM21 was grown for 5 passages and then tested for contamination with strains carrying the *mpn397/relA* wild type allele by PCR, using the same primer pairs used for the primary isolation. Chromosomal DNA from the wild type served as a negative control. As can be seen in Fig. 3.2 (A), strain GPM21 is homogeneous. To verify that only one single transposon insertion is present in the genome of GPM21, chromosomal DNA was digested with BglII and analyzed by Southern blot using a probe against the *aac-ahpD* gentamycin resistance cassette present on the transposon. BglII cuts several hundred times in the genome of *M. pneumoniae* and once in the transposon, thereby ensuring that the gentamycin resistance cassette is always located at the end of one of the resulting fragments. Wild type chromosomal DNA served as a negative control, the transposon delivery vector pMT85 as a positive control. As can be seen in Fig. 3.2 (B), a single band is visible for strain GPM21, confirming a single transposon insertion in the genome of this strain.

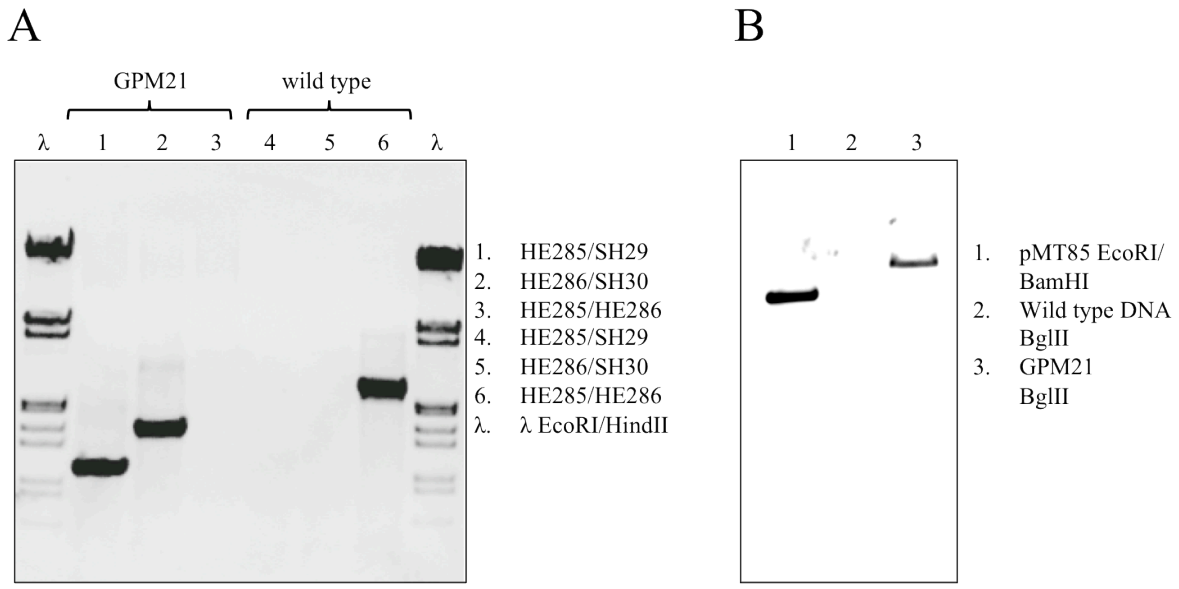


Fig. 3.2: Verification of strain GPM21

A: Test PCR to verify homogeneity of strain GPM21; B: Southern blot analysis of GPM21 to verify the single transposon insertion in the genome. GPM21 is a homogeneous strain with a single transposon insertion between bases 904/905 *of mpn397/relA*.

mpn241/whiA

A single clone harbouring a transposon insertion in Mpn241/WhiA was identified in position E10.11-2 and isolated from the library. The PCR fragments obtained from this strain using primer primers HE324/SH29 and HE325/SH30 were sequenced and aligned with the wild type sequence of *mpn241/whiA*. The transposon inserted after base 260 in respect to the first nucleotide of the gene and therefore met the Glass criteria. However, the transposon insertion

generated a duplication of the first 8 nucleotides upstream of the insertion site, which now can also be found downstream of the insertion. This duplication results in the formation of an alternative ORF downstream of the insertion site with TTG as a possible start codon. The possible ORF consists of 197 codons from the newly formed start TTG to the native stop codon TAA of the gene, leaving the conserved sequences for the conserved WhiA domain unaffected (see Fig. 3.3). Even though it is unclear if the transposon insertion disrupted the gene or just shortened the open reading frame, the strain was renamed GPM22 and used for further studies that might answer this question.

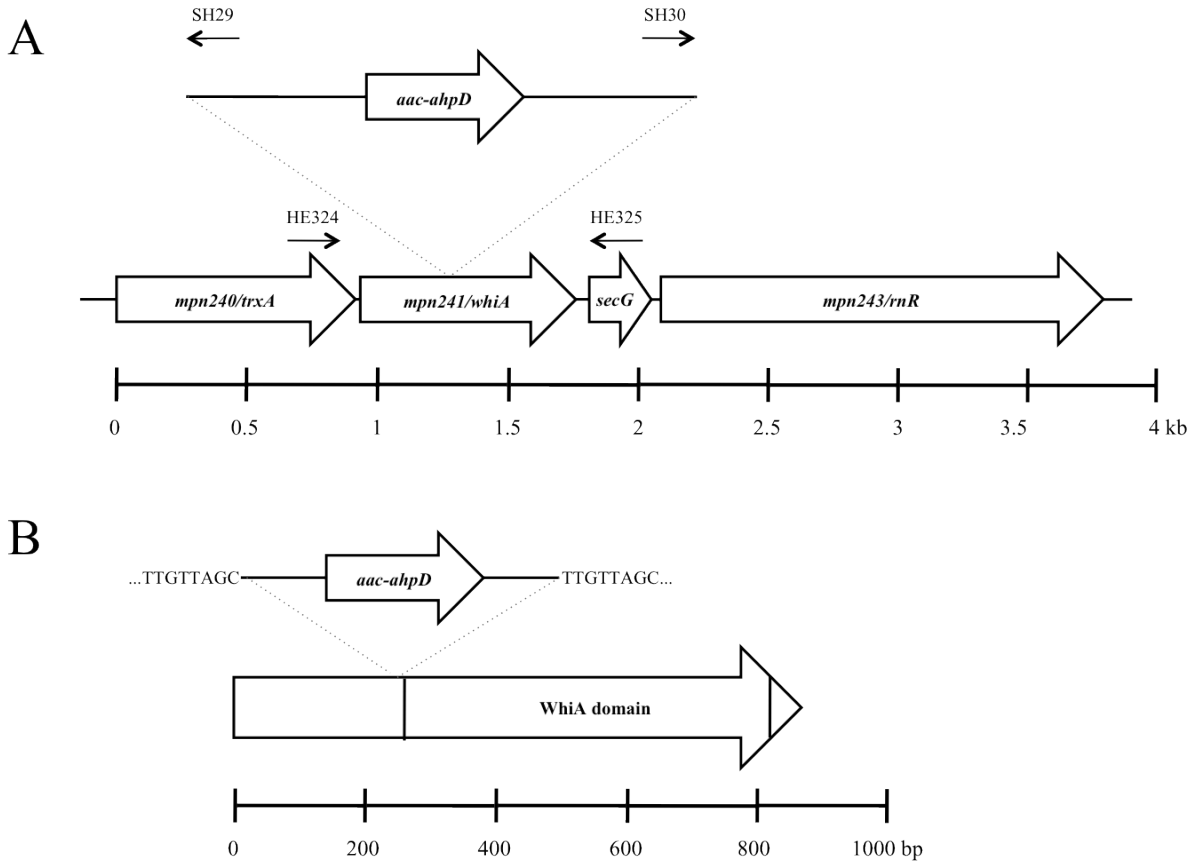


Fig. 3.3: Isolation of a *mpn241/whiA* transposon insertion mutant.

A: Schematic overview of the screen and orientation of the transposon insertion; B: Schematic overview of the transposon insertion in strain E10.11-2. The duplication of the sequence TTGTTAGC results in the formation of an alternative ORF downstream of the insertion site, using TTG from this sequence as a possible start codon.

Strain GPM22 was grown for 3 passages and tested for contaminations with the *mpn241/whiA* wild type allele by PCR, using the same primer pairs used for the primary screen. Because such contaminations were detected, the strain was spread on MP agar plates containing gentamycin. Single colonies were picked after 14 days, transferred in liquid medium and again grown for 3 passages and tested by PCR. 9 of 11 clones were free from contamination,

and two of these were grown to higher passages and tested by Southern blot for single transposon insertions. These experiments finally confirmed that GPM22, after re-isolation, is a homogeneous strain harbouring a single transposon insertion in Mpn241.

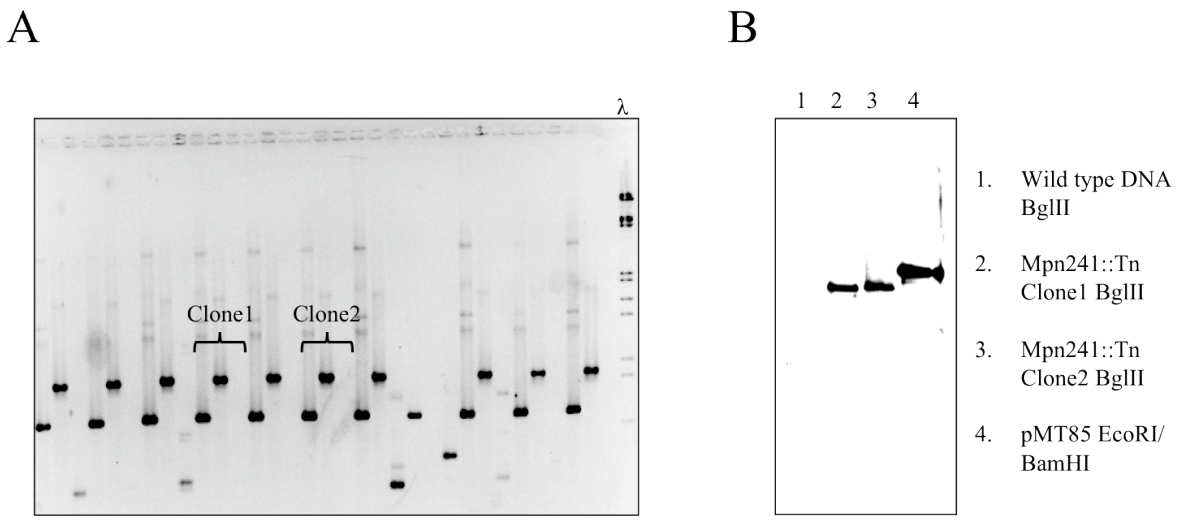


Fig. 3.4: Verification of strain GPM21

A: Colony PCR from 11 single clones from clone E10.11-2. The PCR fragments resulting from primer couples SH29/HE324 confirm the disruption of *mpn241/whiA*. B: Southern blot analysis of clone1 and clone2 of E10.11-2 confirms a single transposon insertion in strain GPM22.

3.2 SPICE: An approach to identify binding sites of DNA-binding proteins *in vivo*

A common method to determine the binding sites of DNA binding proteins in eukaryotes are ChIP-chip or ChIP-seq, chromatin immuno-precipitation combined with tiled microarray chips (-chip) or high throughput sequencing (-seq). In principle, the DNA binding protein is covalently bound to its binding site by chemical cross-linking, the cells are lysed, DNA fragmented and the protein-DNA complexes (the chromatin) immuno-precipitated with specific antibodies against the protein of interest. The DNA bound to the protein is finally recovered and further analyzed using the methods mentioned above.

Aside from eukaryotes, this method has also been applied to study trafficking of transcription units during transcription initiation or to map promoters in *E. coli* (Mooney *et al.*, 2009; Herring *et al.*, 2005). In addition, a modified method referred to as ChAP-chip (chromatin-affinity precipitation combined with tiling microarrays) has been used to determine the binding sites of DnaA in *B. subtilis in vivo* (Ishikawa *et al.*, 2007). In contrast to ChIP, cross-linked chromatin is isolated by affinity purification using tagged versions of the DNA binding protein that are expressed *in vivo*. Since this method theoretically can be applied to every

DNA-binding protein in bacteria, the idea was to use it to identify the binding sites of the essential, putative transcription regulators in *M. pneumoniae*. To achieve this, *M. pneumoniae* strains expressing STREP-tagged transcription regulators were constructed and a protocol to cross-link and purify protein-DNA complexes should be developed.

3.2.1 Proof of principle: *B. subtilis* GltC

Using STREP-tagged protein baits to purify cross-linked protein has been published for *B. subtilis* GltC and other proteins, a method referred to as SPINE (Commichau *et al.*, 2007; Herzberg *et al.*, 2007). GltC is the transcriptional activator of the *gltAB* operon known to bind to its operator sequence in the presence of glucose and ammonium with concomitant absence of external glutamate, thereby enhancing the expression of the glutamate synthase GltAB. Because a *B. subtilis gltC::spc* strain, a complementation plasmid expressing a STREP-tagged GltC and a cross-linking protocol for *B. subtilis* are available in our lab, GltC was chosen as a proof of principle.

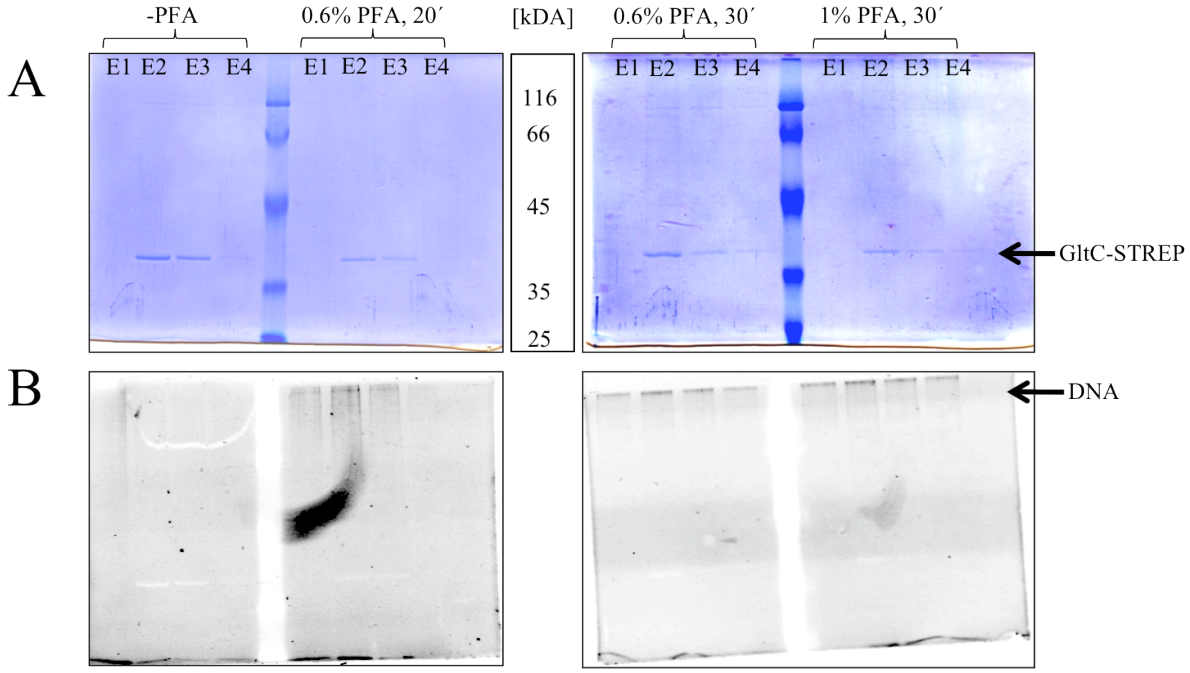


Fig. 3.5: Co-purification of DNA cross-linked to GltC

A: confirmation of GltC-STREP purification after cross-linking by SDS-PAGE; B: ethidium bromide staining of the same gels reveals cross-linked, co-purified DNA as faint smears in samples treated with para-formaldehyde (PFA) but not in the untreated sample. The dark spot on the left picture is an artefact.

A pre-culture of *B. subtilis* GP738 (*gltC::spc*) complemented with plasmid pGP951 was grown overnight in LB medium and used to inoculate two times 1 l of CS-glucose medium.

Cross-linking was performed according to the SPINE protocol (Herzberg *et al.*, 2007) with slight modifications: cells were incubated with 0.6% para-formaldehyde for 20 min and 30 min, and with 1% para-formaldehyde for 30 min to find the optimal conditions for protein-DNA cross-linking. An untreated sample was kept as a negative control. Cells were disrupted by using a OneShot manifold and sonicated to fragment the DNA. Purification of GltC was verified by SDS-PAGE and in addition examined for the presence of DNA by ethidium bromide staining of the PAA gels (see Fig. 3.5, see above). DNA was visible in samples treated with para-formaldehyde. Elution fractions containing protein-DNA complexes were pooled, the volume reduced and the cross-linking reversed by overnight incubation at 65°C. The DNA was recovered by using the QiaQuick PCR Purification kit. For qualitative validation of specific co-purification of the *gltAB* promoter, the recovered DNA was used as template in a PCR using primers IW01 and IW02 that specifically amplify the region containing the GltC binding site. Primers MZ01 and MZ02 that amplify *crh* were chosen as a negative control because of similar chemical properties, same fragment size and distance of *crh* to *gltAB* (about 1560 kbp).

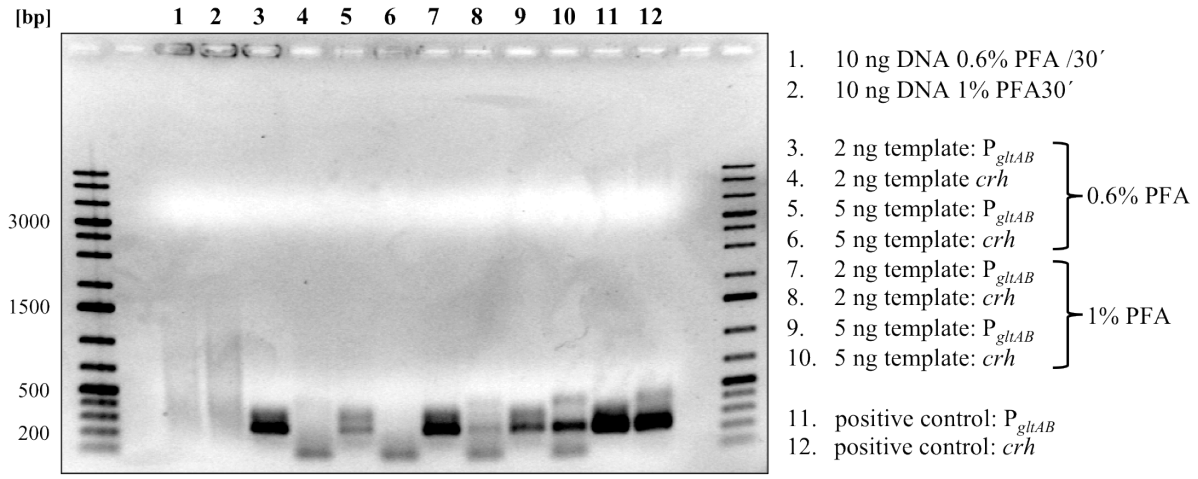


Fig. 3.6: PCR analysis of recovered DNA from GltC

Qualitative analysis of the ChAP-chip experiment with GltC. Recovered DNA has an average size of 500 bp (lanes 1 and 2); the *gltAB* promoter (P_{gltAB}) can be amplified from recovered DNA, whereas *crh* fragment cannot (lanes 3-8). Extended cross-linking (1% PFA, 30´) leads to contamination with DNA other than P_{gltAB} (lanes 8 and 9). Chromosomal DNA as a template served as a positive control (lanes 11 and 12).

The results of this experiment are summarized in Fig. 3.6. The promoter of the *gltAB* operon can specifically be co-purified with GltC and recovered, whereas the negative control cannot. This experiment suggests that targets of any transcription regulator can be identified by this

method. In analogy to the adapted SPINE technique and to later applications, i.e. analysis of co-purified DNA using microarray chips, the experimental design was named SPICE, the SPINE on a chip experiment.

3.2.2 Applying SPICE to *M. pneumoniae*

To apply the SPICE technique to *M. pneumoniae*, strains expressing STREP-tagged transcription regulators had to be constructed. Therefore, the genes *mpn124/hrcA*, *mpn239/gntR*, *mpn241/whiA* and *mpn329/fur* were cloned between the NotI/EcoRI or NotI/SacI restriction sites of the expression vector pClpB-TAP which has been used to express modified genes in *M. pneumoniae* previously (Kühner *et al.*, 2009). The sequence for a C-terminal STREP-tag followed by two stop codons was added to each gene with the respective reverse primer. The resulting plasmids pGP1455 (HrcA-STREP), pGP1456 (Mpn239-STREP), pGP1457 (Mpn329-STREP) and pGP1458 (Mpn241-STREP) were then used to transform *M. pneumoniae* M129.

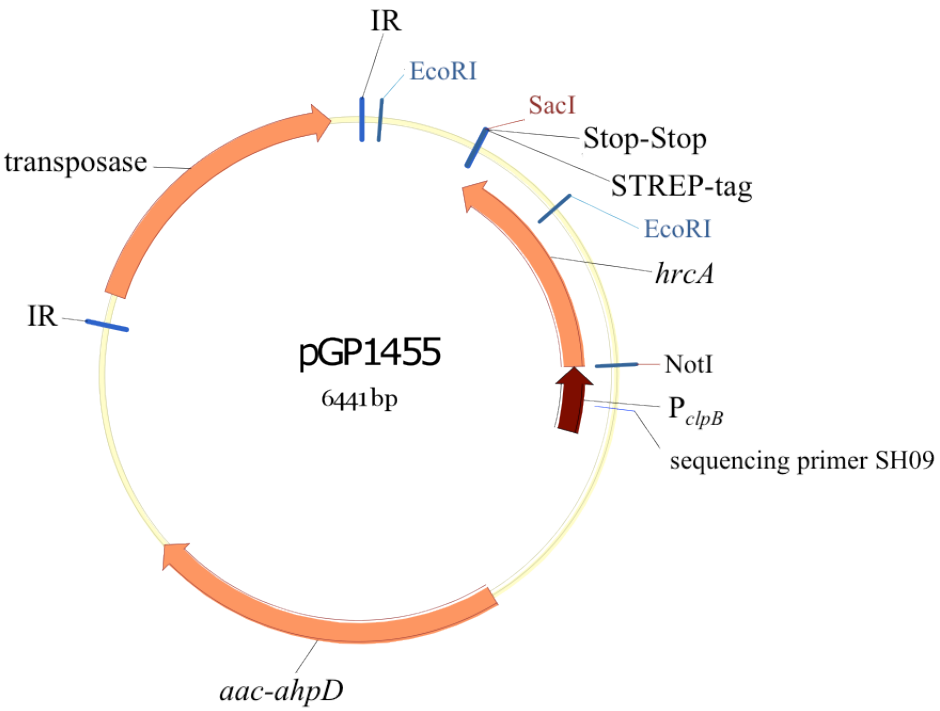


Fig. 3.7: Transposon delivery vector pGP1455

Plasmid pGP1455 is shown as an example for the over-expression vectors constructed. The vectors integrate once into the genome and allow expression of genes under the control of the *clpB* promoter.

M. pneumoniae strains transformed with plasmids pGP1455-1458 were spread on MP agar plates to obtain single colonies. Four colonies from each transformation sample were picked, grown for three passages and then tested by PCR for the integration of the transposon using primer SH09 and the respective reverse primer of each gene (Fig. 3.8, below). Two clones of these four were named and used for further experiments (see appendix, Tab. 7.16).

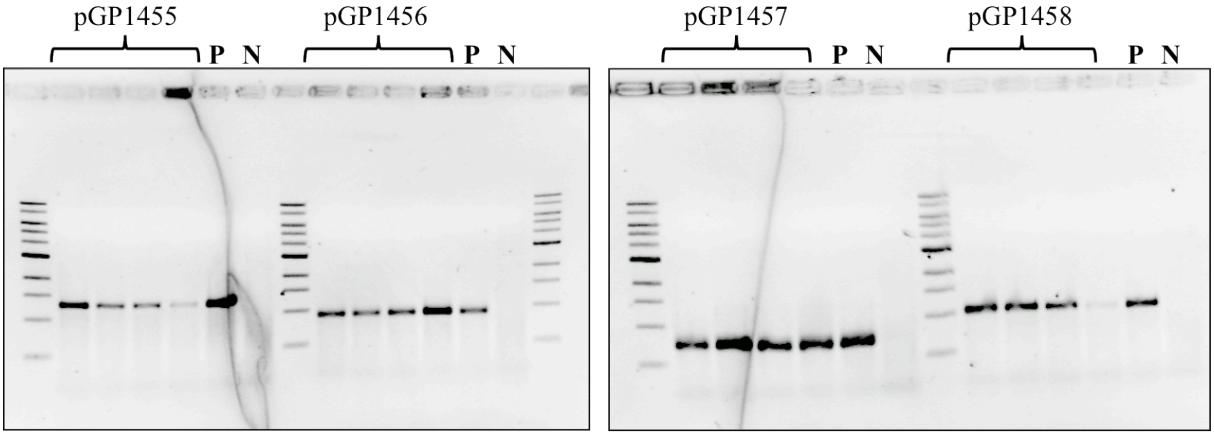


Fig. 3.8: Verification of the inserted promoter-gene fusions in by PCR

Test PCR for the detection of integrated transposons. The artificial promoter-gene fusion could be detected in all strains and in the positive control (P), but not in wild type cells that served as a negative control (N). The 1 kb DNA ladder from Fermentas was used as a size standard.

After growing strains GPM13/14 (expreesing HrcA-STREP), GPM15/16 (expressing Mpn239-STREP), GPM17/18 (expressing Mpn329-STREP) and GPM19/20 (expressing Mpn241-STREP) to the fifth passage, all strains were investigated by Western blot for the expression of the STREP-tagged proteins. An antibody against *M. pneumoniae* GlpK that also detects STREP-tagged proteins was used for this analysis (Hames *et al.*, 2005). *M. pneumoniae* strain GPM78 expressing STREP-tagged HPrK served both as a positive control and an internal standard to determine the size of detected signal (Schmidl *et al.*, 2007). Only Mpn124/HrcA-STREP and Mpn241/WhiA-STREP were detectable by Western blotting of 12% (see Fig. 3.9) and 16% SDS-PAGE (not shown), even though all proteins are expressed under the control of the same promoter, suggesting post-transcriptional regulation. In addition, only Mpn124/HrcA-STREP, Mpn239/GntR-STREP and Mpn241/WhiA-STREP could be purified from *M. pneumoniae* extracts of two litres of culture (10x 300 cm^2), with Mpn239/GntR-STREP and Mpn241/WhiA-STREP in little amounts only detectable by Western blotting (Fig. 3.10).

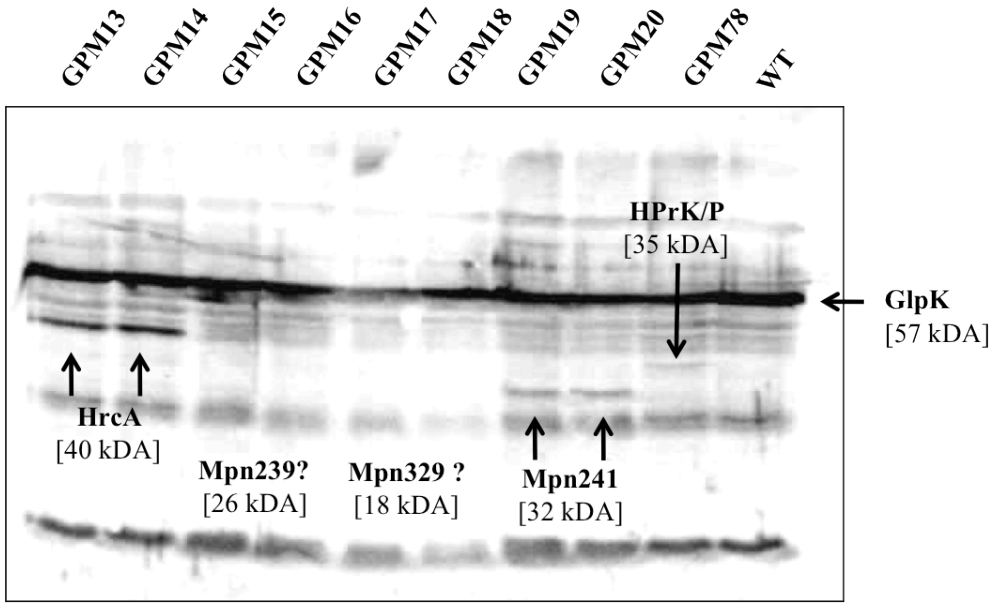


Fig. 3.9: Detection of STREP-tagged transcription regulators in GPM13 to GPM20

Western blot of a 12% SDS-PAGE to detect STREP-tagged proteins in strains GPM13 to GPM20. GlpK (57 kDa) is visible in all strains; in addition, signals of tagged proteins are visible in strains GPM13/14, GPM19/20 and in the positive control GPM78. No signals are visible in extracts of GPM14/16 and GPM17/18.

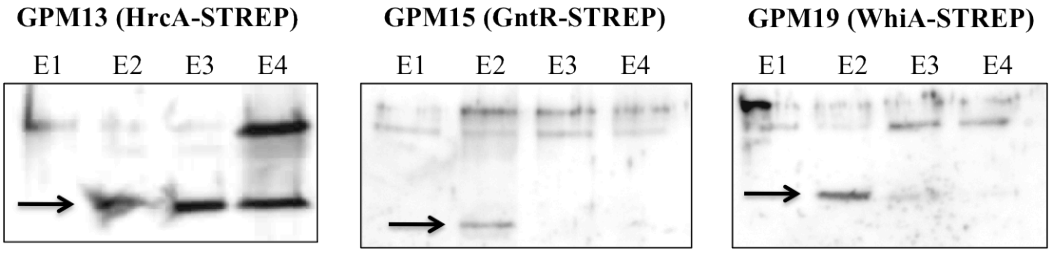


Fig. 3.10: Test-purification of STREP-tagged proteins from strains GPM13, GPM15 and GPM19.

All three proteins were detected in the elution fractions resulting from STREP purification; only minor amounts of Mpn239/GntR-STREP and Mpn241/WhiA-STREP could be purified from extracts of GMP15 and GPM19, respectively. 2 l of culture (10x 300 cm^2) were used as starting material.

Strain GPM13 was used to develop a cross-linking protocol. Since the target genes of HrcA in *M. pneumoniae* have been described before, their promoters can be used as a proof of principle for the functionality of the SPICE technique in this organism (Chang *et al.*, 2008). To detect specific up-concentration of *M. pneumoniae* DNA fragments carrying the CIRCE element by co-purification with HrcA-STREP after cross-linking, primers for qPCR were designed to amplify promoter regions of *lon* and *dnaK*. The promoters of these genes harbour perfect CIRCE elements, and the PCR primers were designed to amplify DNA fragments including these sequences. Primers pairs that amplify the promoter regions of *ldh* and *ackA*

were designed to serve as a negative control to calculate the ratio of specific and unspecific DNA cross-linked to HrcA (Halbedel *et al.*, 2007). GPM13 was grown at 37°C for 96 h, washed with PBS and then cross-linked by directly incubating adherent cells at 37°C with 0.5% PFA or scraped and incubated with 0.5% PFA while shaking. Purification of HrcA-STREP was controlled by Western blot analysis and co-purified DNA quantified after recovering. Cross-linking longer then 20 min eliminated the possibility to purify HrcA-STREP from extracts of GPM13. 5 ng and 10 ng of the recovered DNA were analyzed by qPCR to calculate the ratios of specific (P_{lon}/P_{dnaK}) and unspecific bound DNA (P_{ldh}/P_{ackA}).

	P_{dnaK}/P_{ldh}	P_{dnaK}/P_{ackA}	P_{lon}/P_{ldh}	P_{lon}/P_{ackA}
Experiment 1	0,6	0,9	1,5	2,3
Experiment 2	0,6	1,0	1,7	2,7
Experiment 3	0,8	1,2	1,8	2,5
Experiment 4	6,6	1,5	20,5	4,8
Experiment 5	1,3	1,0	20,8	15,3

Tab. 3.2: Results of SPICE experiments with GPM13

Ratios of specific to unspecific bound DNA cross-linked to HrcA, calculated from the results from qPCR of 5 individual experiments. No significant up-concentration of DNA specifically bound by HrcA (P_{lon} and P_{dnaK}) was achieved.

According to the current literature, a 30-fold enrichment of specifically bound DNA has to be achieved by ChIP/ChAP for downstream analysis such as tiling arrays or high throughput sequencing. The highest enrichment of expected DNA fragments achieved in these experiments was about 20 times. Even though this is close to the minimal enrichment required it lacks reproducibility, was only achieved for one of the two expected fragments and might therefore also be artificial (see Tab. 3.2 above).

In summary, no optimized SPICE protocol like that for *B. subtilis* could be developed for *M. pneumoniae*. In addition, the inefficient purification of other transcription regulators from *M. pneumoniae* strains GPM15/16 and GPM19/20 made SPICE experiments impossible for those proteins. Moreover, due to the experimental effort and the running costs for medium and material, the SPICE project on *M. pneumoniae* was stopped.

3.3 Experiments aiming to identify targets of Mpn329/Fur

Fur and other members of the Fur family require Fe^{2+} ions that are bound by conserved histidines to dimerize and to obtain DNA-binding activity (Herbig and Helmann, 2001; Jacquamet *et al.*, 2009). These histidines are also conserved in the protein encoded by *mpn329/fur* of *M. pneumoniae*. However, this gene is essential in *M. pneumoniae,* thereby preventing the identification of target genes of this regulator by comparative proteome or transcriptome analysis. Thus, the question was raised if genes under control of this regulator can be identified if cells are starved starved iron. To achieve this, cells were grown in the presence of the iron chelator 2,2'-dipyridyl which has been used to induce iron starvation in bacteria previously, and were then analyzed by proteome or microarray analysis (Baichoo *et al.*, 2002; Merrel *et al.*, 2003).

3.3.1 Proteome analysis of *M. pneumoniae* grown in the presence of 2,2'-dipyridyl

A growth experiment probing different concentrations of 2,2'-dipyridyl was performed to determine the MIC in Hayflick medium containing glucose. A final concentration of 1 mg/ml was found to inhibit growth of *M. pneumoniae*. Therefore, a final concentration of 0.5 mg/ml was chosen to induce iron starvation in the following experiments.

In the first set of experiments, cells were grown for 96 h in the presence or absence of 2,2'-dipyridyl, lysed in ZAP buffer and centrifuged. The supernatant (the soluble fraction) was removed and the pellet resolved in 6 M urea. After a second centrifugation step the supernatant (the insoluble fraction) was collected. 8 µg of both fractions were separated by SDS-PAGE and silver stained. Extracts of untreated wild type cells and cells that have been grown in the presence of an equal amount of the solvent served as controls. Differences in the protein composition are marked in Fig. 3.11 (no. 1 to 8). No differences between untreated cells and the solvent control were observed, whereas some bands occur in higher or lower intensity in extracts of cells grown in the presence of 2,2'-dipyridyl. The bands were excised from the gel and the proteins analyzed by mass spectrometry. Amounts of proteins Mpn394/Nox, Mpn120/GrpE and Mpn517 were elevated in treated cells. Peptides of proteins Mpn314/MraZ and Mpn254/CinA were found in a fourth band that showed higher intensity in extracts of treated cells. However, it is unclear if only one or both identified proteins are more abundant in the sample. Furthermore, proteins were identified in bands of lower intensity, namely Mpn555/TriggerC and peptides of Mpn321/DhfR and Mpn323/NrdI in a second band.

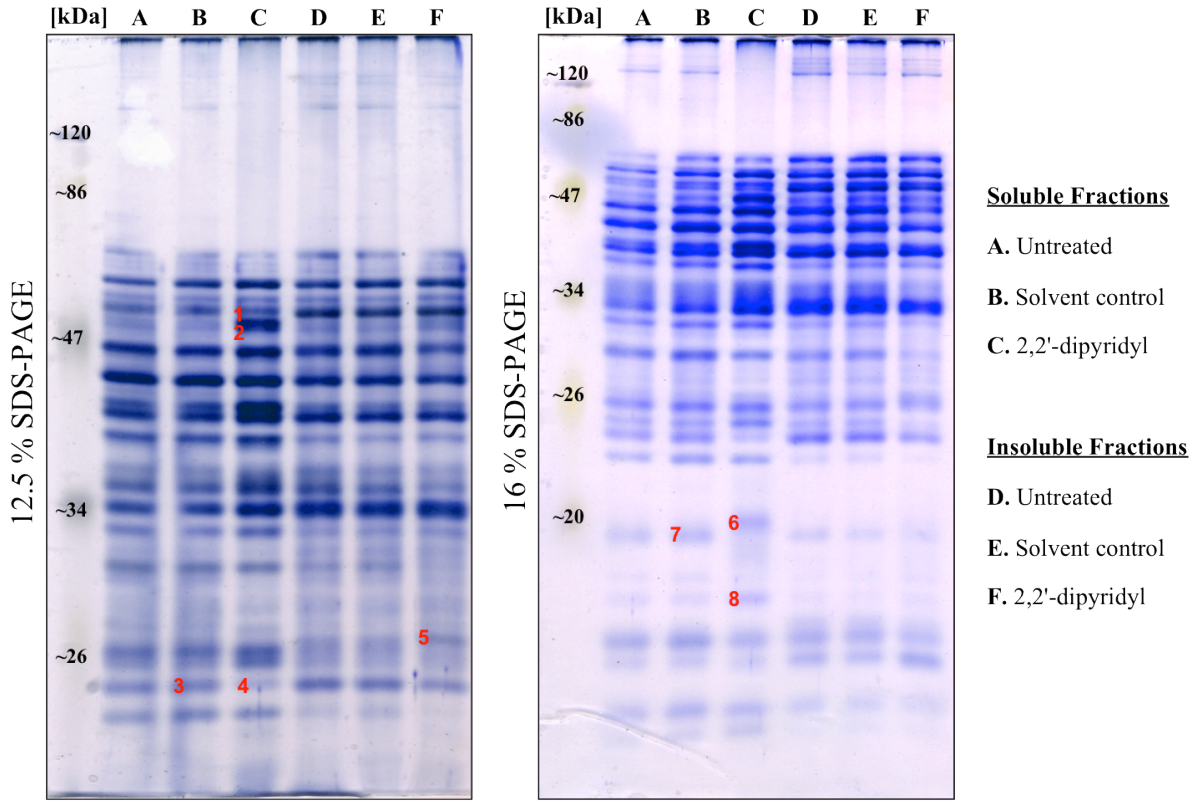


Fig. 3.11: Comparison of the proteome of cells grown in the presence or absence of 2,2'-dipyridyl

Silver stained SDS-PAGE of soluble and insoluble fraction of *M. pneumoniae* grown in the presence or absence of iron chelator 2,2'-dipyridyl. The color of the pictures was changed for better visibility of the bands. Differences in the proteome are marked with numbers; 1 and 2: Mpn394/Nox; 3 and 4: Mpn555/TriggerC; 5: Mpn120/GrpE; 6: Mpn517; 7: Mpn321/DhfR and Mpn323/NrdI; 8: Mpn314/MraZ and Mpn254/CinA.

Since all Fur-family transcription regulators described in the *Firmicutes* are acting as repressors, only genes that are up-regulated during iron starvation are regarded as possible targets of Mpn329/Fur in this experiment. The heat shock protein Mpn120/GrpE is encoded in the second gene of the *mpn119/dnaJ* operon that is controlled by Mpn124/HrcA (Chang *et al.*, 2009). Therefore it is unlikely that Mpn329/Fur might regulate this gene. *mpn321/dhfR* and *mpn323/nrdI* are both located in the *thyA* operon consisting of 5 genes involved in nucleotide synthesis. Because this pathway is not related to any function regulated by Fur-family transcription regulators in other *Firmicutes*, i.e. metal ion homeostasis or oxidative stress, they were not further considered. ORFs *mpn394/nox* and *mpn517* are coding for a NADH oxidase and a putative flavin mononucleotide dependent reductase, respectively. Thus, both enzymes are putatively involved in regulating the redox-state of the cell. Since Mpn329/Fur shows some similarity with PerR from *B. subtilis* (see introduction) and the mechanism how *M. pneumoniae* protects itself from self-induced oxidative stress by H_2O_2 formation is

unknown it was anticipated that these genes might be involved in an oxidative stress response and are under control of Mpn329/Fur. To verify this hypothesis, Slot blot experiments with RNA from treated and untreated cultures were performed to determine if the regulatory effect observed in the proteome is also visible on the transcription level.

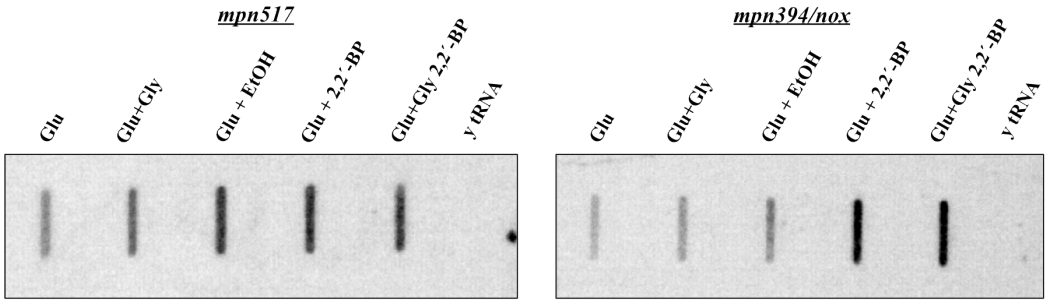


Fig. 3.12: Slot blot analysis of RNA from cells grown on the presence or absence of 2,2'-dipyridyl.
Whereas transcription of *mpn517* was almost stable under different conditions, transcription of *mpn394/nox* was elevated in cells upon iron depletion. Glu: glucose; Gly: glycerol; EtOH: 50% ethanol (solvent); 2,2´-BP: 2,2'-dipyridyl.

Transcription of *mpn394/nox* was enhanced in cultures grown in the presence of 2,2'-dipyridyl, but not in cultures that were grown in the presence of equal amounts of the solvent or in untreated cells. In addition, this effect was independent of the carbon source available. On the other hand, transcription of *mpn517* was found to be almost stable in the same cultures (Fig. 3.12).

To test if Mpn329/Fur directly regulates transcription of *mpn394/nox* by binding to its promoter, Mpn329/Fur was cloned in plasmid pWH844, over-expressed in *E. coli* and purified by His-tag affinity purification. Purified Mpn329/Fur was used for EMSA (electrophoretic mobility shift assays) with DNA fragments of the 5´ regions of *mpn394/nox*, *mpn517* and *mpn329/fur* since members of this regulator family are often auto-regulated. None of the three DNA fragments shifted after the addition of increasing amounts of purified Mpn329/Fur, showing that no protein-DNA interaction occurred *in vitro* (Fig. 3.13). EMSA experiments with the same DNA fragments were also performed with purified Mpn239/GntR to exclude the involvement of this protein in the regulatory events observed. As expected, no interaction of Mpn239/GntR with any of the three DNA fragments was observed (data not shown). Therefore, the regulation of *mpn394/nox* in response to iron starvation seems not to be mediated by Mpn329/Fur or Mpn239/GntR.

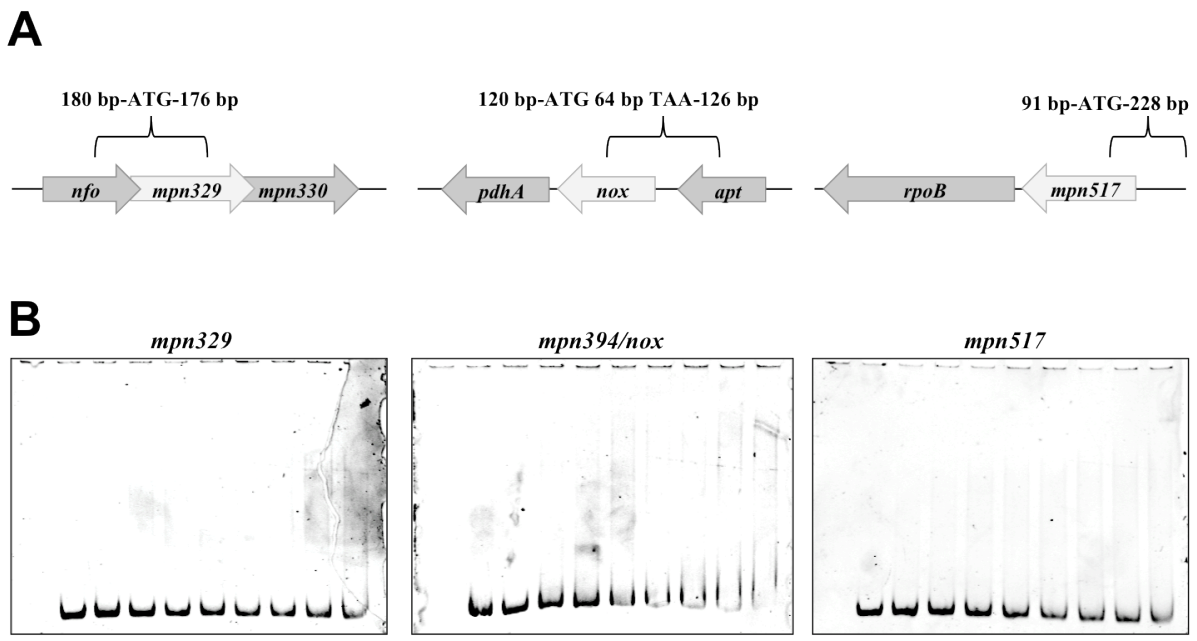


Fig. 3.13: EMSA to proof Mpn329/Fur-DNA interactions

A: DNA fragments used for EMSA. Start (ATG) and stop (TAA) codons of neighboured genes are indicated. **B:** EMSA with DNA fragments harbouring the putative promoters of *mpn394/nox*, *mpn517* and *mpn329/fur* and purified Mpn329/Fur. No protein-DNA interaction is observed *in vitro*.

3.3.2 Probing *M. pneumoniae* promoters in *B. subtilis*

In vitro approaches to confirm protein-DNA interactions are difficult if the interaction is mediated by unknown cofactors such as ions or metabolites. To overcome this problem, the idea to establish a screening system for *M. pneumoniae* promoters in a related heterologous system such as *B. subtilis* was developed. Promoter activities of putatively regulated genes could then be probed by *lacZ* fusions in *B. subtilis* strains expressing or lacking the putative transcription regulators.

To test *M. pneumoniae* promoters that are putatively regulated by Mpn329/Fur in *B. subtilis*, *B. subtilis* Δ*fur* Δ*perR* double mutants were constructed that either express or lack Mpn329/Fur (Tab. 7.15, appendix). These strains were then transformed with transcriptional *lacZ* fusions of the *mpn517* and *mpn394/nox* promoters and tested for ß-galactosidase activity. Enzymatic activities were lower than 5 Miller units in all strains tested, suggesting no transcription from these promoters. Since *M. pneumoniae* promoters are only weakly conserved and lack sequences of -35 regions described in other bacteria (Weiner *et al*., 2000), the promoter sequences were mutated to obtain optimal -35 regions. However, these strains also lack ß-galactosidase activity.

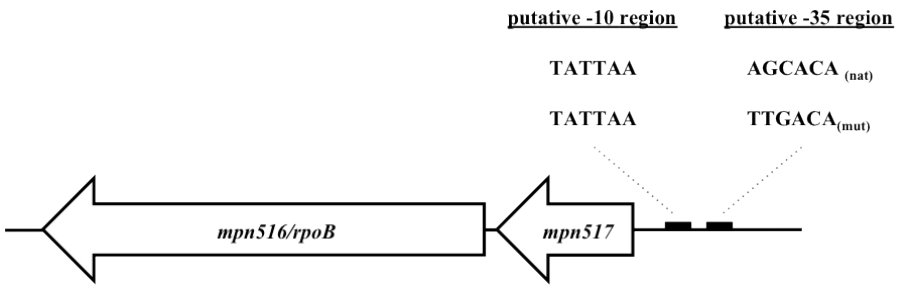


Fig. 3.14: Mutation of the -35 regions of putative promoters

Putative promoters of *mpn517* and *mpn394/nox* were mutated by CCR to achieve optimized -35 regions recognized by sigma70 factors. -35 regions are required to initiate transcription in *B. subtilis* but are dispensable in *M. pneumoniae* (Halbedel *et al.*, 2007). TATTAA has been shown to function as -10 region in *M. pneumoniae* in previous works (Weiner *et al.*, 2000). (*nat*): native promoter; (*mut*): mutated promoter with optimized -35 region)

The reason for this might be general differences in promoter recognition or transcription initiation due to differences in conserved amino acid compositions in conserved regions of the SigA from *M. pneumoniae* (see discussion).

To test this hypothesis, *B. subtilis* strains harbouring transcriptional *lacZ* fusions of *mpn394/nox*, *mpn517* and *ldh* promters (Halbedel *et al.*, 2007) were transformed with a plasmid that allows expression of SigA from *M. pneumoniae* to achieve transcription from the these promoters. This kind of experiment has been used previously to allow transcription from *Rhizobium etli* promoters in *E. coli* (Ramirez-Romero *et al.*, 2006). Because cloning of *M. pneumoniae sigA* into expression vector pBQ200 was not working in *E. coli*, *B. subtilis* strains were directly transformed with the ligation sample. Resulting plasmid pGP283 was then isolated from *B. subtilis* and controlled by sequencing. Again, no ß-galactosidase activity was detected in any of these strains expressing SigA from *M. pneumoniae*.

In summary, *B. subtilis* is not able to recognize and/or to initiate transcription from *M. pneumoniae* promoters, suggesting fundamental differences in requirements for promoter recognition in these two bacteria (see discussion). In addition, expression of *M. pneumoniae* SigA in *B. subtilis* does not allow transcription from these promoters. Therefore, *B. subtilis* is not suitable as a heterologous host to probe *M. pneumoniae* promoters.

Strain	Genotype	Miller Units
GP869	*perR::spc, fur:mls, gltA::P_{degQ}-mpn329*	0,7
GP870	*perR::spc, fur:mls, gltA::pGltA-Kan*	3,7
GP871	*perR::spc, fur:mls, gltA::P_{degQ}-mpn329, amyE ::$P_{Mpn517(nat)}$-lacZ*	0,8
GP872	*perR::spc, fur::mls, gltA::pGlt-Kan, amyE ::$P_{Mpn517(nat)}$-lacZ*	1,1
GP873	*perR::spc, fur::mls, gltA::P_{degQ}-mpn329,amyE ::$P_{nox(nat)}$-lacZ*	0,2
GP874	*perR::spc, fur:mls, gltA::pGlt-Kan, amyE ::$P_{nox(nat)}$-lacZ*	1,0
GP875	*perR::spc, fur:mls, gltA::P_{degQ}-mpn329, amyE ::$P_{Mpn517(mut)}$-lacZ*	1,4
GP876	*perR::spc, fur::mls, gltA::pGlt-Kan, amyE ::$P_{Mpn517(mut)}$-lacZ*	1,0
GP877	*perR::spc, fur::mls, gltA::P_{degQ}-mpn329,amyE ::$P_{nox(mut)}$-lacZ*	1,4
GP878	*perR::spc, fur:mls, gltA::pGlt-Kan, amyE ::$P_{nox(mut)}$-lacZ*	1,1
GP880	*amyE ::$P_{517(nat)}$-lacZ*	0
GP880 +	*amyE ::$P_{517(nat)}$-lacZ,* [P_{deqQ}-*mpn352/sigA*]	
pGP283		0
GP882	*amyE ::$P_{ldh(nat)}$-lacZ*	0
GP882 +	*amyE ::$P_{ldh(nat)}$-lacZ* [P_{deqQ}-*mpn352/sigA*]	
pGP283		0
GP278	*trpC2 amyE::(xynP-lacZ cat) xylR::Erm^R*	376,4

Tab. 3.3: Activities of *M. pneumoniae* promoters in *B. subtilis*

ß-galactosidase activities (in Miller units) in *B. subtilis* strains harbouring transcriptional *lacZ* fusions of *M. pneumoniae* promoters. Plasmid pGP283 was used to express $SigA_{Mpn}$ in *B. subtilis*. Strain GP278 (Singh *et al.*, 2007) served as a positive control. *nat*: native promoter; *mut*: promoters with optimized -35 regions.

3.3.3 Microarray analysis of *M. pneumoniae* grown in the presence of 2,2'-dipyridyl

Proteome analyses of different bacterial mutants or the comparison of the proteome from treated and untreated cells allow studying the output of all regulations that might occur in the different strains. However, proteome analyses lack information about the level at which the regulations occur and if the regulations observed are direct or indirect effects. In addition, excreted, insoluble or proteins with extreme size or pI are often not recognized, leaving a gap in the global analysis. To study global transcription in response to certain stimuli or in different strains, microarrays or, until recently, deep transcriptome sequencing is used. Since no target of Mpn329/Fur was identified by studying the proteome of cells treated with iron chelator 2,2'-dipyridyl, microarrays were performed using the same conditions.

M. pneumoniae was grown for 96 h in pre-culture in Hayflick medium containing glucose and then seeded in the same medium with or without 2,2'-dipyridyl. Samples were taken after 6/24/48/72/96 h. In theory, transcription of target genes of Mpn329/Fur should be constantly up-regulated along the growth curve in cells that grow in the presence of the chelator due to the lack of ferric iron, the cofactor of Fur-family proteins required for DNA binding. However, global transcription patterns of treated and untreated cells after 48 h showed fundamental differences making comparisons impossible for the later time points. Therefore, only time points 6 h, 24 h and 48 h were compared. Several changes in transcription were observed in cells treated with the iron chelator, but only a small set of genes exhibited a constant up-regulation compared to untreated cells.

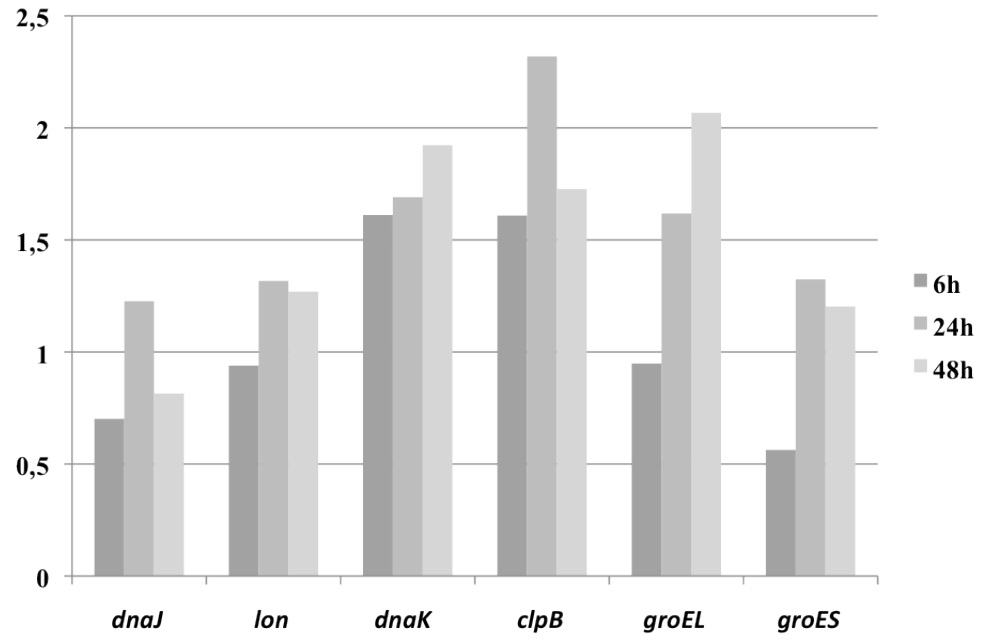


Fig. 3.15: Induction of the HrcA regulon upon iron depletion.

Fold changes of the HrcA operon in cells grown in the presence of 2,2´-dipyridyl. Transcription of the whole operon is constantly up-regulated upon iron depletion.

Among those genes was the whole HrcA regulon, consisting of *dnaK*, *groEL/groES*, *dnaJ*, *lon* and *clpB* and genes located downstream of these genes. Induction of the HrcA regulon was reported for several stresses beside heat shock in *M. pneumoniae* (Güell *et al.*, 2009). Interestingly, genes harbouring a conserved CIRCE element in their promoter region showed the highest regulations, whereas genes with cryptic CIRCE elements (such as *dnaJ*) only showed minor up-regulation (Fig.3.15).

A second group of genes constantly up-regulated in cells treated with 2,2'-dipyridyl was the *oppB* operon, consisting of genes coding for four subunits of a putative peptide transporter and two ribosomal proteins. As for the HrcA regulon, up-regulation of transcription of the *oppB* operon in response to several different conditions was already reported previously (Güell *et al.*, 2009).

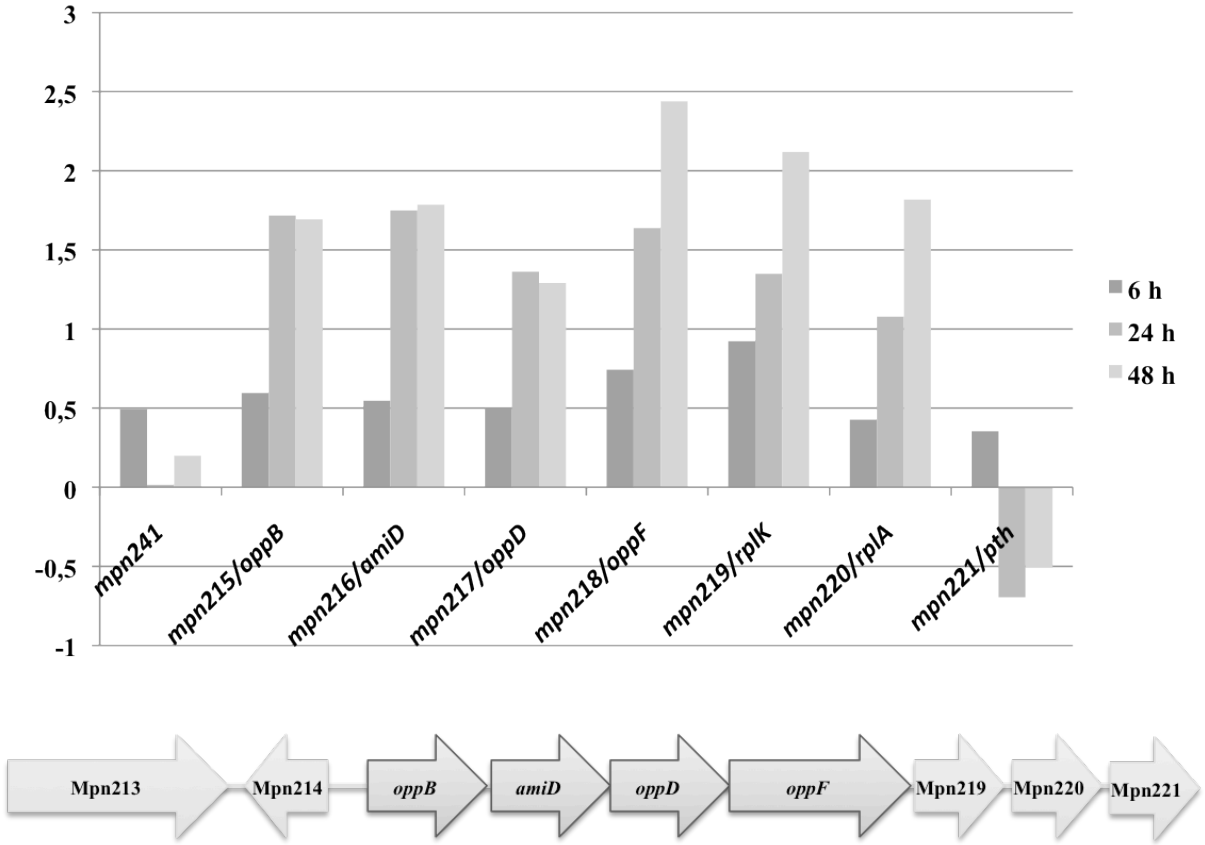


Fig. 3.16: Induction of the *oppB* operon upon iron depletion

Fold changes of genes of the *oppB* operon and neighbouring upon iron depletion. Below: Scheme of the *oppB* operon. The whole *oppB* operon (*oppB* to Mpn220) is constitutively up-regulated in cells grown in the presence of 2,2´-dipyridyl whereas neighbouring genes are not.

Finally, the array data was analyzed for transcriptional changes of genes identified in the proteome analysis described in chapter 3.3.1. In good agreement with the data obtained from Slot-blot experiments, no up-regulation of transcription of *mpn517* was observed. Transcription of *mpn394/nox* was only slightly elevated after 6 h but increased constantly during the next time points, also confirming prior experiments. However, sequence analysis of the promoter regions of genes up-regulated upon iron depletion and their orthologues in *M. genitalium* showed that none of them harboured a common DNA motif in their promoter region. Moreover, none of the promoters contained DNA sequences resembling Fur or PerR

boxes described in other bacteria. Thus, it is unlikely that Mpn329/Fur directly regulates these genes and the mechanisms leading to their enhanced transcription requires further investigation.

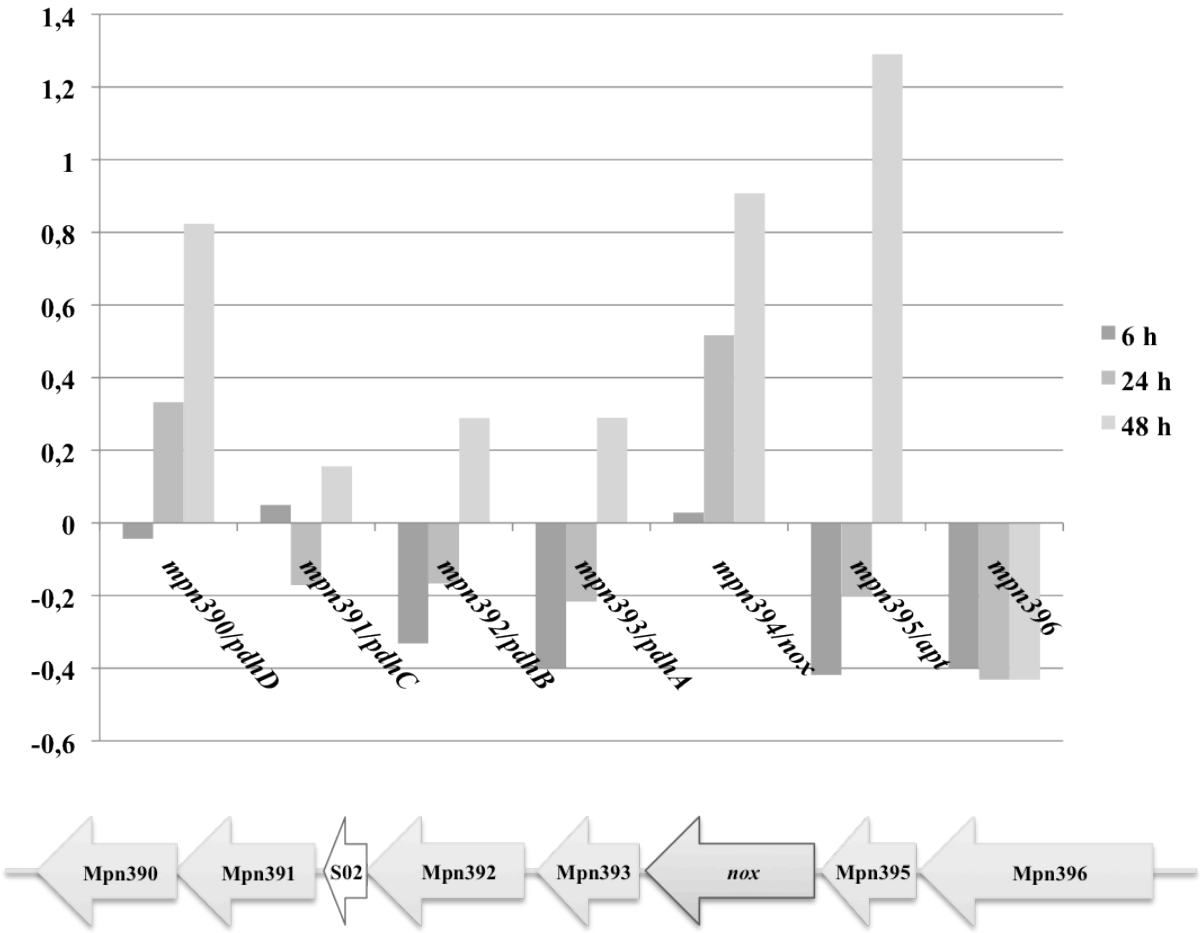


Fig. 3.17: Increasing transcription of *mpn394/nox* upon iron depletion

Fold changes of *nox* and neighbouring genes. S02 is a putative small RNA not considered in the microarray analysis. *nox* shows adjacent transcription patterns compared to neighbouring genes.

Since no protein encoded in the *oppB* operon was identified in the proteome analysis described in chapter 3.3.1, an alternative method was applied allowing the comprehensive analysis of all proteins from *M. pneumoniae* by SDS-PAGE without the separation of soluble and insoluble fractions.

For this analysis, *M. pneumoniae* wild type, GPM21 (*mpn397/relA::Tn*) and GPM52 (*glpD::Tn*) were grown in the presence or absence of 2,2'-dipyridyl and directly lysed in protein loading buffer. GPM52 served as a general control, and GPM21 was chosen to exclude the possibility that probable regulatory effects observed are mediated by (p)ppGpp (Miethke *et al.*, 2006).

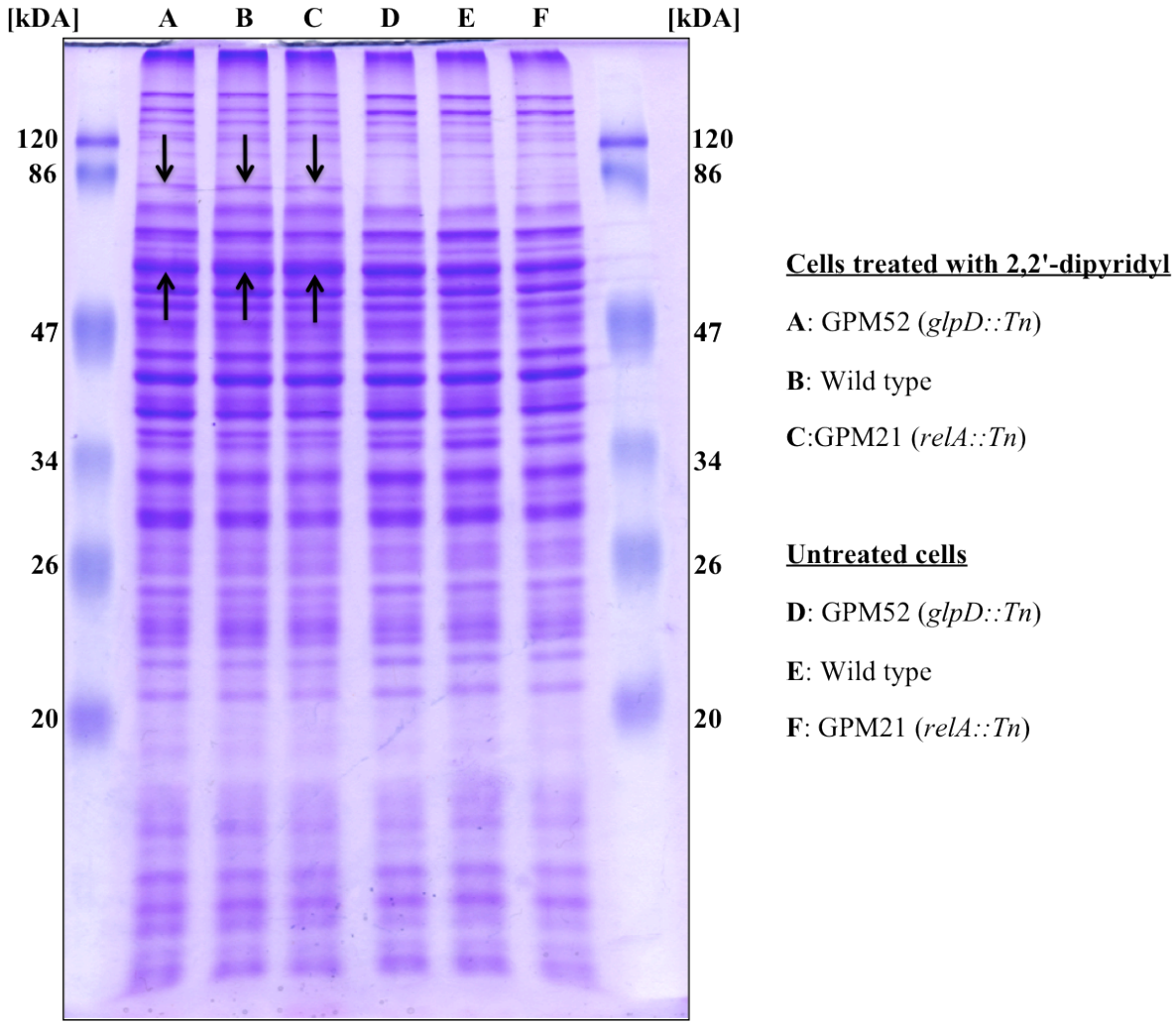


Fig. 3.18: Proteome analysis of *M. pneumoniae* strains grown in the presence or absence of 2,2'-dipyridyl
Complete proteome of *M. pneumoniae* wild type, GPM52 and GPM21 treated or untreated with 2,2'-dipyridyl. Elevated levels of OppF (upper band indicated) and PtsI (lower band indicated) were found in all three strains when treated with the iron chelator. No enhanced levels of Nox are visible by this method, probably due to the lack of separation of soluble and insoluble protein factions.

Only two bands showing elevated protein amounts were observed in samples from all cultures treated with 2,2'-dipyridyl. MS analysis identified these proteins as PtsI (fold change of 1.5) and OppF (fold change of 3.5). No up-regulation of *ptsI* transcription was found in the microarray analysis. Therefore, the finding of elevated amounts of PtsI might be a secondary effect due to a modified stability of the protein. On the other hand, transcriptome and proteome data of the *oppB* operon correlate well, indicating that this operon indeed is up-regulated during iron depletion due to a direct mechnism that still needs to be explored. The up-regulation of the *oppB* operon might have been overlooked in the first proteome analysis (chapter 3.3.1) due to the fact that these proteins are anchored in the membrane of *M. pneumoniae*. Thus, they might have got lost during the separation of soluble and insoluble

fractions. Transcription of other genes identified in the proteome analysis (chapter 3.3.1) either showed no or opposed regulations compared to the proteomic data. Thus, transcriptome and proteome data do not necessarily correlate in *M. pneumoniae* (as described previously: Halbedel, 2006), underlining the importance of coupling both approaches.

3.4 Experiments aiming to characterize the function of Mpn266

When this work started, only three putative transcription regulators were described in *M. pneumoniae*: Mpn124/HrcA, Mpn239/GntR and Mpn329/Fur. Transcription regulator Mpn266/Spx was missanotated as an arsenate reductase and its homology to *B. subtilis* Spx was discovered during this work. *B. subtilis* Spx is an unusual transcription regulator because its does not make direct contact with DNA. Instead, Spx mediates conformational changes of the RNA polymerase holoenzyme by interacting with RpoA C-terminal domain, thereby allowing it to bind to additional, *cis* acting elements upstream of promoters (Reyes and Zuber, 2008). Expression of Spx in *B. subtilis* is tightly controlled at the level of transcription. In addition, the protein has a notably short half-life due to its C-terminal amino acid sequence that mediates immediate proteolysis by the protease ClpXP.

3.4.1 Proof of interaction of Mpn266/Spx with RpoA

To proof whether Mpn266/Spx really is an Spx orthologe, interaction of Mpn266/Spx with RpoA, the alpha subunit of *M. pneumoniae* RNA polymerase was studied. The genes coding for Mpn266/Spx and Mpn191/RpoA were cloned into vectors of an adenylate cyclase based bacterial-two-hybrid system (B2H), expressed in *E. coli* and colonies examined for ß-galactosidase activity as an indicator of protein-protein interactions. *B. subtilis* Spx and RpoA served as a positive control.

As expected, a strong interaction of *B. subtilis* RpoA with Spx was found (coordinates A3-A4 and C1-C2 in Fig. 3.19). In addition, both *B. subtilis* and *M. pneumoniae* RpoA interact with themselfes as already known from crystal structures of the RNA polymerase from *T. aquaticus* (Zhang *et al.*, 1999). Surprisingly, no interaction between *B. subtilis* and *M. pneumoniae* RpoA is observed even though these proteins are highly conserved in all bacteria. A weak interaction of Mpn266/Spx is visible with Mpn191/RpoA (H5), demonstrating that this protein behaves similar its orthologe Spx in *B. subtilis*. Furthermore,

Mpn266/Spx interacts with RpoA from *B. subtilis* providing evidence that the amino acid residues required for this interaction are conserved in the *M. pneumoniae* protein (C5 and E3).

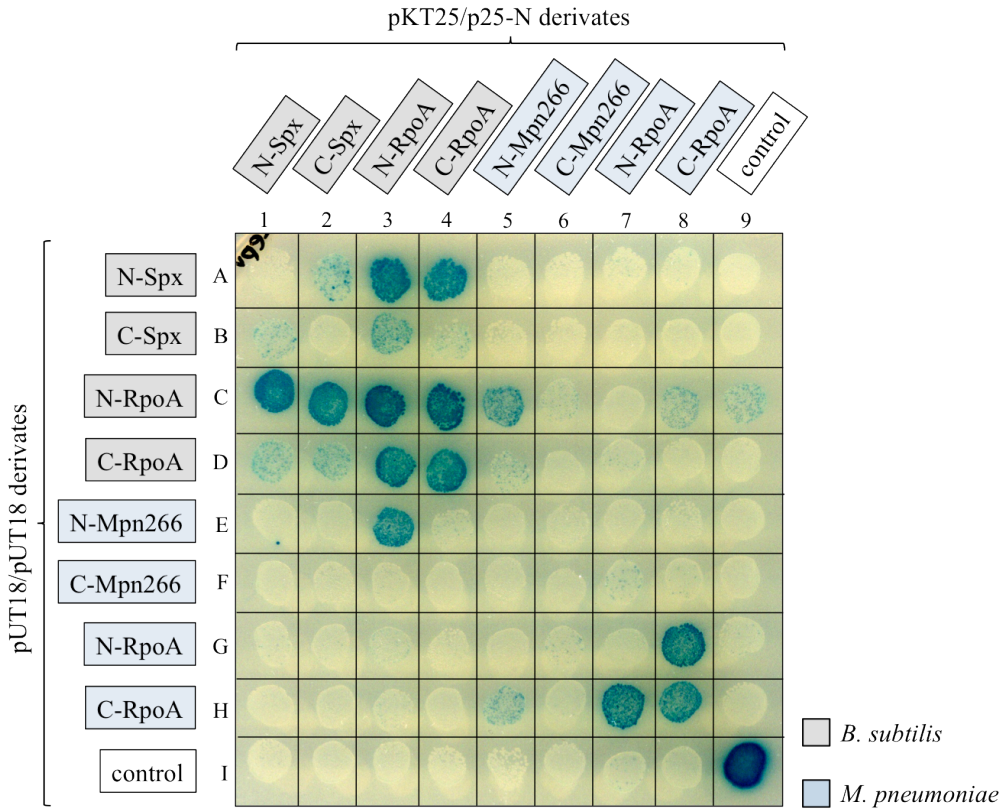


Fig. 3.19: Interaction of Spx and Mpn266 with RpoA from *M. pneumoniae* and *B. subtilis*

Scanned picture of the B2H experiment. N- and C-terminal fusions of probed proteins with the T18 and T25 domain of the *B. pertussis* adenylate cyclase in the respective vectors are indicated. The experiment provides evidence that Mpn266 indeed interacts with the C-terminal domain of Mpn191/RpoA *in vivo*.

3.4.2 Complementation of *B. subtilis* Δ*spx* with Mpn266/Spx

Since Mpn266/Spx is a putative orthologe of *B. subtilis* Spx, the question was raised if Mpn266/Spx could complement a *B. subtilis* Δ*spx* strain. To test this, Mpn266/Spx was cloned into plasmid pGP382 that allows expression of the gene with a C-terminal STREP-tag in *B. subtilis*. The resulting plasmid pGP1454 was used to transform *B. subtilis* Δ*spx*. Only one single colony resulted from this transformation whereas >200 colonies were achieved in a parallel sample using the same amount of empty vector pGP382, which served as a positive control. To proof that this strain does not harbour suppressor mutations, the chromosomal *rpoA* gene and the plasmid were sequenced again and expression of Mpn266/Spx in this strain verified by test purification using the STREP-tag. No mutations were found and Mpn266/Spx

could be purified from extracts of the strain (not shown). However, the possibility of suppressor mutations in other loci cannot be excluded.

B. subtilis 168, *B. subtilis* Δ*spx* and *B. subtilis* Δ*spx* carrying either the empty expression vector pGP382 or complementation plasmid pGP1454 were probed in an agar disc diffusion assay for their sensitivity against the chemical compound paraquat. Paraquat uncouples the oxidative phosphorylation and leads to the intracellular formation of hydrogen peroxide, thus to oxidative stress, and *B. subtilis* Δ*spx* was reported to be highly sensitive (You *et al.*, 2008).

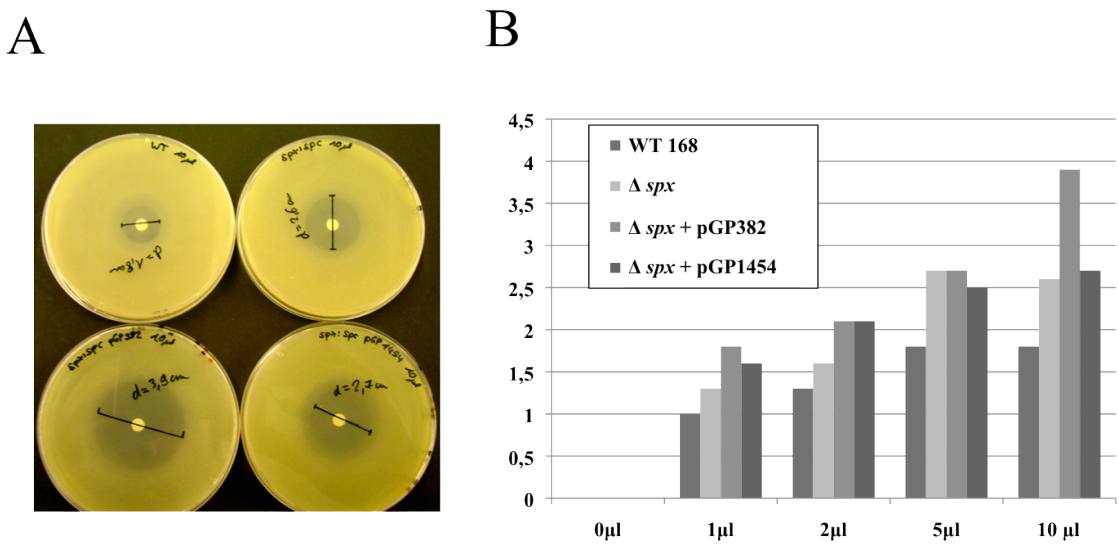


Fig. 3.20: Outcome of the disc diffusion experiment.

A: Sample picture of the experiment. *B. subtilis* wild type (top left), Δ*spx* (top right), Δ*spx* with pGP382 (bottom left) and Δ*spx* with complementation plasmid pGP1454 treated with 10 µl paraquat solution. B: Diameters of the bacteria-free areola caused by increasing amounts of paraquat solution (10 mg/ml).

As can be seen in Fig. 3.20, *B. subtilis* Δ*spx* indeed shows an elevated sensitivity towards increasing amounts of paraquat in this experiment. However, complementation plasmid pGP1454 did not restore resistance to paraquat to the wild type level. This is either due to the fact that the protein Mpn266/Spx itself is not capable to complement the *spx* deletion in *B. subtilis*, or because the unregulated expression of Mpn266/Spx leads to unknown suppressor mutations in *B. subtilis* Δ*spx* after transformation with pGP1454.

At the time these experiments were performed, a contemporary publication about protein interactions in *M. pneumoniae* and the identification of Mpn266/Spx as a putative transcription regulator was announced (Kühner *et al.*, 2009; Anne-Claude Gavin, personal communication). Therefore, no further experiments concerning this transcription regulator and its role in *M. pneumoniae* were performed.

3.5 Experiments aiming to characterize the role of Mpn397/RelA

Whereas no mutants of other transcription regulators could be isolated, several transposon insertions were found in *mpn397/relA* (chapter 3.1.2). The availability of mutants allows performing several experiments to characterize the role of this gene and to characterize a phenotype of the mutant.

3.5.1 Analysis of conserved domains in Mpn397/RelA

RelA homologues are conserved in all *Mycoplasmas* for which a genome sequence is available, with the exception of the members of the *M. hominis* group. *M. pneumoniae* Mpn397/RelA has 32% amino acid identity (E value: 2e-63) with *B. subtilis* RelA which has been characterized previously. In addition, the conserved RxKD motif present in bi-functional RelA protein, i.e. RelA proteins that possess both (p)ppGpp synthetase and hydrolase activity, is present in Mpn397/RelA, although as HxKD. In monofunctional proteins such as *E. coli* RelA, an ExDD motif replaces these amino acid residues (Sajish *et al.*, 2007; Sajish *et al.*, 2009). Furthermore, the HD superfamily hydrolysis domain is conserved in the N-terminus of the protein as described for all bi-functional Rel proteins (Aravind and Koonin, 1998). This domain was shown to hydrolyse (p)ppGpp in a Mg^{2+} dependent manner (Sajish *et al.*, 2009). Since no other proteins with (p)ppGpp synthetase activity are encoded in the genome of *M. pneumoniae*, Mpn397/RelA is the only protein in this organism putatively responsible for the synthesis and hydrolysis of (p)ppGpp (Nanamiya *et al.*, 2008).

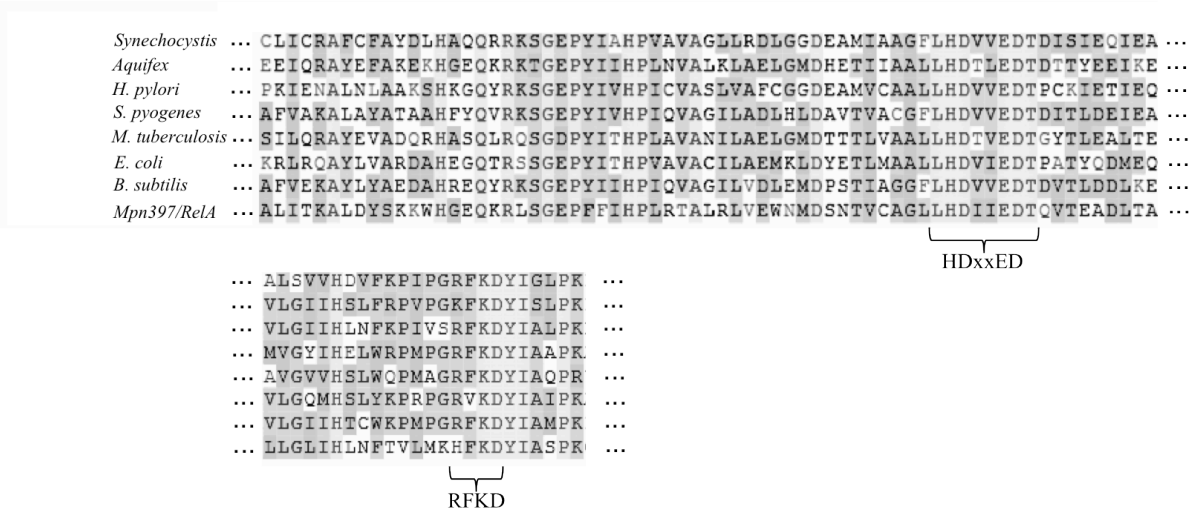


Fig. 3.21: Partial multiple alignment of bi-functional RelA proteins

Amino acid sequences from conserved domains of RelA from *Synechocystis sp., Aquifex sp., H. pylori, S. pyogenes, M. tuberculosis, E. coli, B. subtilis* and Mpn397/RelA. The conserved amino acids residues of the (p)ppGpp synthesis and hydrolysis domain are indicated.

3.5.2 Detection of (p)ppGpp and other metabolites in *M. pneumoniae*

Mpn397/RelA harbours two conserved domains that resemble the (p)ppGpp synthesis and hydrolysis domains of bi-functional RelA enzymes. However, the conserved RFKD motif located in the synthesis domain is present as HFKD in Mpn397/RelA. To test whether this and other differences in the amino acid composition of Mpn397/RelA affect its enzymatic activity, *M. pneumoniae* and the Mpn397/RelA mutant GPM21 were tested for their ability to synthesize (p)ppGpp.

Common methods to induce (p)ppGpp synthesis in bacteria are either starving cells for essential amino acids in a defined medium or treating cells with chemicals such as norvaline, serine-hydroxamate or antibiotics like mupirocin that all mimic amino acid starvation. Norvaline is a synthetic amino acid unfeasible for protein synthesis that competes against valine in being loaded to $tRNA_{Val}$. Serine hydroxamate and mupirocin are synthetic and naturally occurring inhibitors of serine and isoleucine tRNA synthethase, respectively. (p)ppGpp can be labelled and detected by growing cells in the presence of (P^{32})-γ-ATP followed by nucleotide extraction and thin layer chromatography. Since radioactive labelling of pathogenic bacteria *in vivo* was not allowed in the facility this work was performed, an alternative method to detect (p)ppGpp had to be used. Recently developed techniques in the research field of metabolomics allow the detection and quantification of nucleotides, amino acids and metabolites in extracts of bacterial cultures by mass spectrometry coupled with liquid or gas chromatography (LC-MS and GC-MS). These techniques have recently been used to study the stringent response in *S. aureus* (Geiger *et al.*, 2010) and were also adapted to detect (p)ppGpp in *M. pneumoniae* during this work.

M. pneumoniae was grown in Hayflick medium with glucose for 96 h. Then, the medium was exchanged with "Hayflick light" (to reduce background in MS; see chapter 2.1.2) with or without mupirocin or serine hydroxamate, cells lysed and nucleotides extracted as described previously. Treatment of cells with mupirocin for up to one hour did not induce formation of (p)ppGpp. However, detectable amounts of the so far unknown and uncharacterized nucleotide ppGp were found instead in samples treated for 90 min (not shown). The source of this nucleotide is unknown and is rather thought to be an artefact of the antibiotic than a specific cellular response towards amino acid starvation. In contrast, ppGpp but no pppGpp could be detected in extracts of cultures that have been treated with serine hydroxamate for 40 min or 50 min in two separate experiments. No (p)ppGpp was found in control samples treated with "Hayflick light" without serine hydroxamate. To exclude the possibility of false negative results, two different methods were used to detect (p)ppGpp in these experiments: (I)

LC-MS as described in Geiger *et al.* (2010) and (II) HILIC modified as described in Bajad *et al.* (2006). The same experiment was performed with Mpn397/RelA disruption mutant GPM21. As expected, no (p)ppGpp was detected in extracts after treatment with serine hydroxamate for up to 60 min (data not shown; M. Liebeke, personal communication). These experiments confirm that the stringent response and the mechanism of regulation of (p)ppGpp synthesis are conserved in *M. pneumoniae* and that Mpn397/RelA is the only enzyme responsible for the synthesis of ppGpp under these conditions.

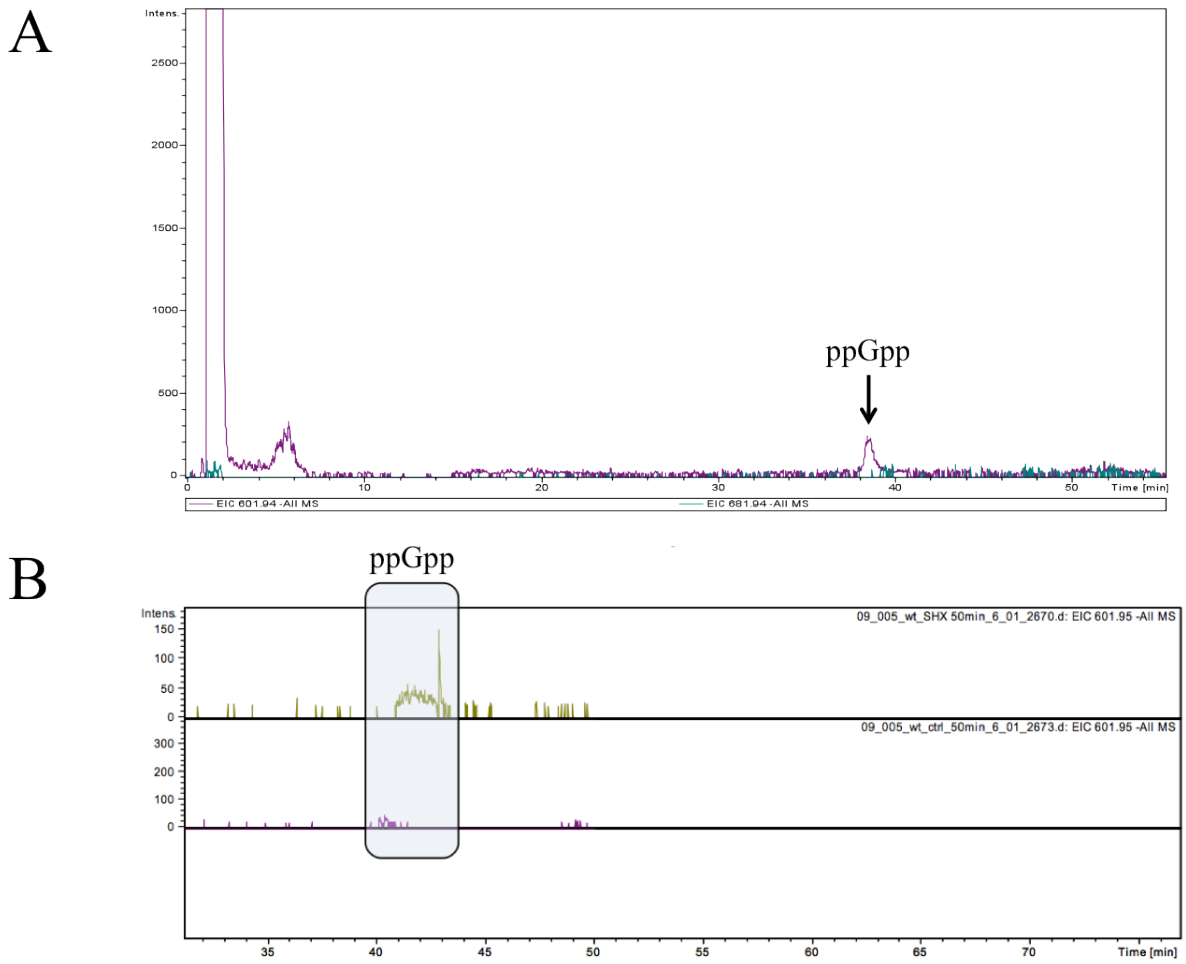


Fig. 3.22: Detection of ppGpp in *M. pneumoniae* by mass spectrometry

Detection of ppGpp in wild type cells treated with serine hydroxamate. A: ppGpp (pink) but no pppGpp (green) were detected after 40 min of treatment using the method described in Geiger *et al.* (2010). B: Only ppGpp was detectable in cells treated for 50 min (yellow signal, top) but not in the untreated control (pink signal, bottom). Here, a modified HILIC approach as described in Bajad *et al.* (2006) was used. No (p)ppGpp was found in *mpn397/relA* mutant strain GPM21 treated with serine hydroxamate (not shown).

Synthesis of (p)ppGpp in the *Firmicutes* is accompanied by a decrease of intracellular GTP due to inhibition of enzymes required for GTP synthesis, and an increase of intracellular ATP due to a so far unknown mechanism. Decrease of GTP is a prerequisite for the down-regulation of rRNA synthesis in *B. subtilis* (Krásný and Gourse, 2004; Natori *et al.*, 2009). *M. pneumoniae* lacks genes coding for enzymes involved in GTP synthesis and is auxotrophic for GTP. Thus, it is tempting to speculate that *M. pneumoniae* is unable to decrease intracellular amounts of GTP in the presence of (p)ppGpp. Quantification of nucleotide mono-, di- and tri-phosphates in samples analyzed by the method described in Geiger *et al.* (2010) indeed provides evidence for this hypothesis: only slight changes of intracellular GTP and ATP occur in *M. pneumoniae* wild type cells in which ppGpp was deteclable. However, this analysis has only been performed once and requires repetition of these experiments.

In addition to the expected nucleotides, another identified compound is worth mentioning. Cyclic adenosine monophosphate (cAMP) functioning as secondary messenger in *E. coli* has been found in extracts of *M. pneumoniae*, even though at low concentrations. This is surprising since no putative adenylate cyclase is present in the genome of *M. pneumoniae.* The finding of cAMP in *M. pneumoniae* extracts could also be confirmed by other workgroups (T. Maier, CRG, Barcelona; personal communication). However, the origin of this compound is so far unknown and its role requires further experiments.

3.5.3 Growth curves of *M. pneumonie* GPM21

Bacterial *relA* mutants show a very characteristic phenotype in a way that these strains are not able to enter stationary phase during growth. Instead, they grow in a log phase-like manner until nutrients are completely depleted from the medium and suddenly starve to death. To analyze the growth behaviour of *M. pneunmoniae* GPM21, growth curves were performed and the wet weight of cultures from this strain compared with the wild type and strain GPM70 as controls. Strain GPM70 harbours a transposon insertion in structural gene *mpn474* and has been described previously (Hegermann *et al.*, 2008). No differences during growth in Hayflick medium containing either glucose or glycerol as carbon sources were observed within these three strains.

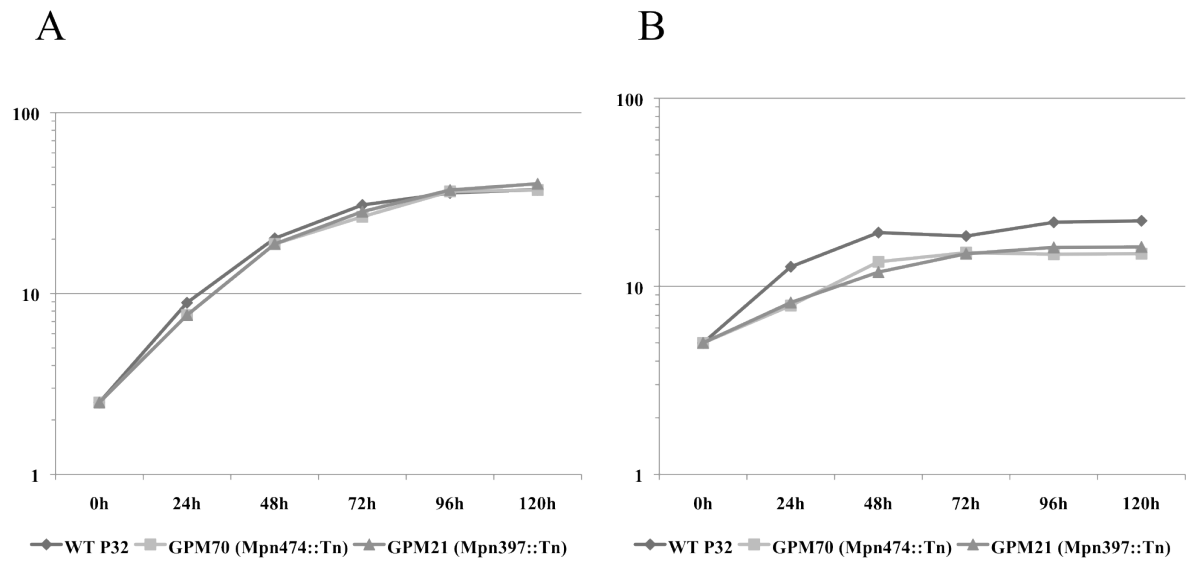


Fig. 3.23: Growth curves of *M. pneumoniae* strains

Growth curves of *M. pneumoniae* wild type, GPM70 and GPM21 determined by the wet weight of cultures grown in Hayflick medium containing either glucose (A) or glycerol (B) as carbon sources.

3.5.4 Scanning electron microscopy of *M. pneumonie* GPM21

(p)ppGpp affects various aspects of bacterial metabolism and also the cell cycle. In addition, (p)ppGpp was found to affect the morphology of some bacteria such as *Mycobacterium smegmatis, Rhizobium etli* or *Helicobacter pylory* (Ojha *et al.*, 2000; Braeken *et al.*, 2008; Zhou *et al.*, 2008). Scanning electron microscopy was performed to determine if disruption of *mpn397/relA* in *M. pneumoniae* GPM21 affects the morphology of the cell. No differences in morphology were observed between GPM21 and the wild type.

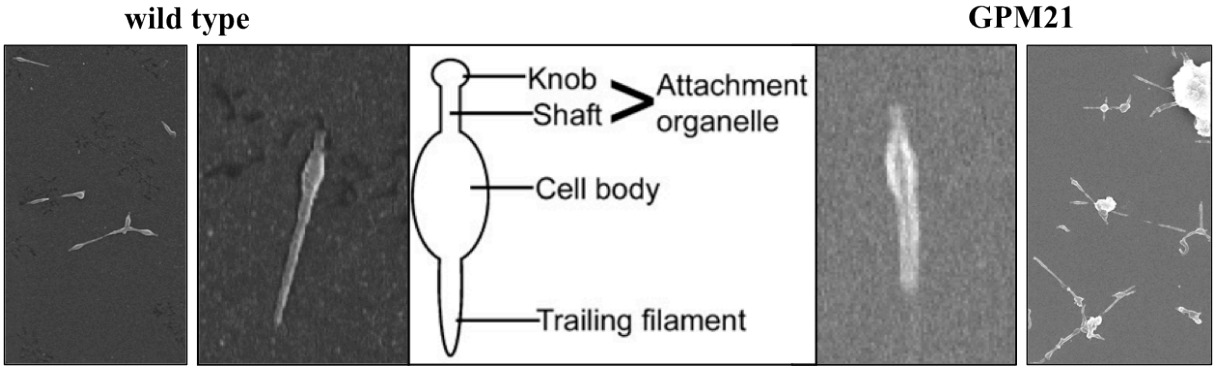


Fig. 3.24: Scanning electron microscopy of *M. pneumoniae* strains

Scanning electron microscopy pictures of *M. pneumoniae* wild type and GPM21. No differences in the morphology of single or dividing cells are observed.

3.5.5 Adaptation of *M. pneumonie* GPM21 to different stresses

Despite nutrient starvation, (p)ppGpp is also involved in stress adaption in bacteria. Implications of (p)ppGpp in the adaption to elevated temperatures, iron depletion, osmotolerance and membrane stress induced by ethanol were reported other bacteria (Varcamonti *et al.*, 2003; Braeken *et al.*, 2008; Vinella *et al.*, 2005; Miethke *et al.*, 2006; Okada *et al.*, 2002; Wei *et al.*, 2004; Yan *et al.*, 2009).

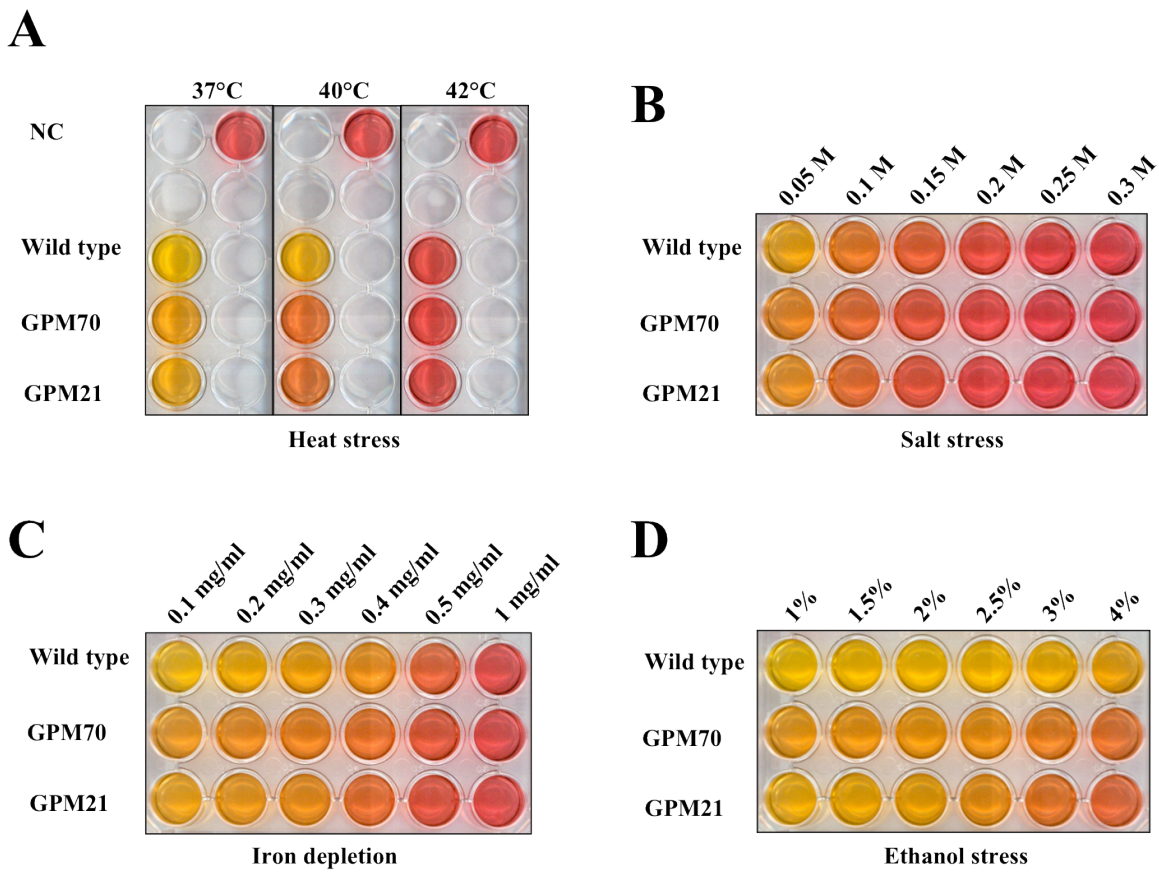


Fig. 3.25: Adaptation of *M. pneumonie* strains to different stresses

Acidification of Hayflick medium by *M. pneumoniae* wild type, GPM79 and GPM21. A: Different temperatures; B Increasing NaCl concentrations; C: Increasing amounts of iron chelator 2,2'-dipyridyl; C: Increasing amounts of ethanol. Ethanol evaporated from the medium. Therefore, this experiment cannot be analyzed.

To test the ability of *M. pneumoniae* wild type and GPM21 to adapt to these stresses, small-scale experiments have been performed. *M. pneumoniae* strains were grown in Hayflick with glucose in microtiter plates for 96 h and the acidification of the medium was monitored by documenting the colour change of the medium from red (basic) to yellow (acidic). The acidification of the medium by lactate or acetate, the end products of glycolysis, correlates with the biomass of cells in the culture. Thus, *M. pneumoniae* strains defective in stress adaptation would grow slower and should therefore acidify the medium less compared to

strains not defective in stress adaptation. Once such a condition is identified, growth curves as described in chapter 3.5.3 would be performed to determine the growth defect by calculating the biomasses of the different strains. However, *M. pneumoniae* wild type, GPM21 and GPM70 (as a control) did not exhibit different acidification patterns in these experiments, suggesting similar abilities to adapt to the stresses tested.

3.5.6 Cytotoxicity of GPM21 towards HeLa and A549 cells

(p)ppGpp is an important regulator that triggers virulence of pathogenic bacteria and adaptation to intracellular growth of facultative intracellular bacteria as shown in various animal infection models (Dalebroux *et al.*, 2010; La *et al.*, 2008). Only very few animal infection models have been reported for *M. pneumoniae*, among them guinea pigs and baboons (Dumke *et al.*, 2004; Hardy *et al.*, 2009). Instead, cytotoxicity assays using human cell lines have been used to determine virulence factors of *M. pneumoniae*, because of their simple experimental setup and the fact that no approval of ethics committees is required (Kannan and Baseman, 2006; Hames *et al.*, 2009; Schmidl *et al.*, 2010).

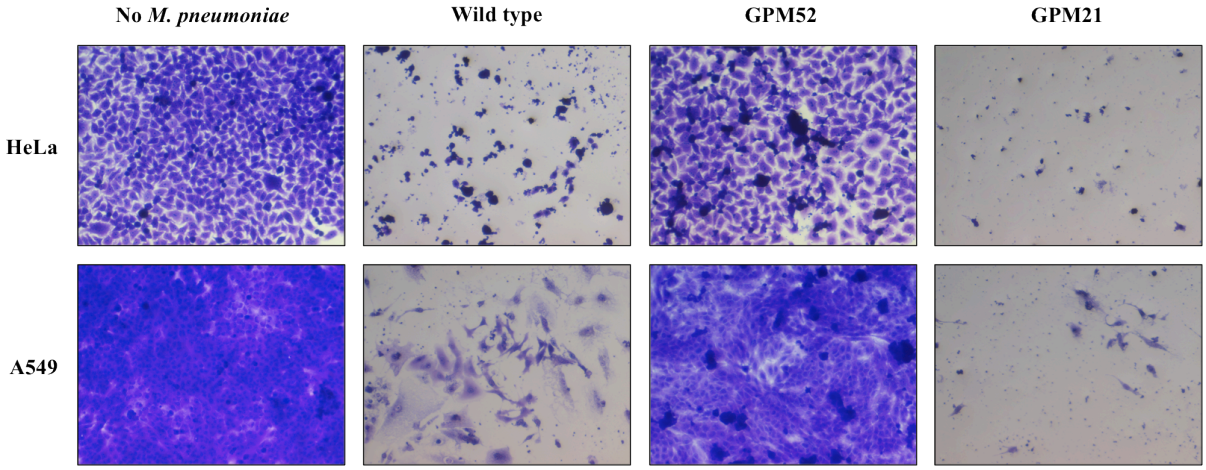


Fig. 3.26: Cytotoxicity of *M. pneumoniae* strains towards HeLa and A549 cells

No differences are observed between *M. pneumoniae* wild type and GPM21, whereas GPM52 is strongly impaired in cytotoxicity

To determine if GPM21 is impaired in cytotoxicity, HeLa cell assays have been performed and cytotoxicity was compared with *M. pneumoniae* wild type and GPM52 (*glpD::Tn*) which was shown to have a reduced cytotoxicity (Hames *et al.*, 2009). In addition to HeLa cells, cytotoxicity towards human alveolar basal epithelial cell line A549 was tested using the same

strains (Lieber *et al.*, 1967). The ability of *M. pneumoniae* to internalise and replicate in this cell line was reported previously (Yavlovich *et al.*, 2004). No differences were observed between *M. pneumoniae* wild type and GPM21, whereas GPM52 was impaired in cytotoxicity towards both cell lines (Fig. 3.26).

The killing assay to determine internalisation described in Yavlovich *et al.* (2004) could not be performed since gentamycine is used as a resistance marker in *M. pneumoniae* mutants. An alternative strategy using the Mynox® *Mycoplasma* Elimination Kit (Minerva Biolabs, Berlin) to kill extracellular *M. pneumoniae* cells failed because the kit was quite ineffective, probably due to the high number of *M. pneumoniae* cells used to infect the host cells (not shown).

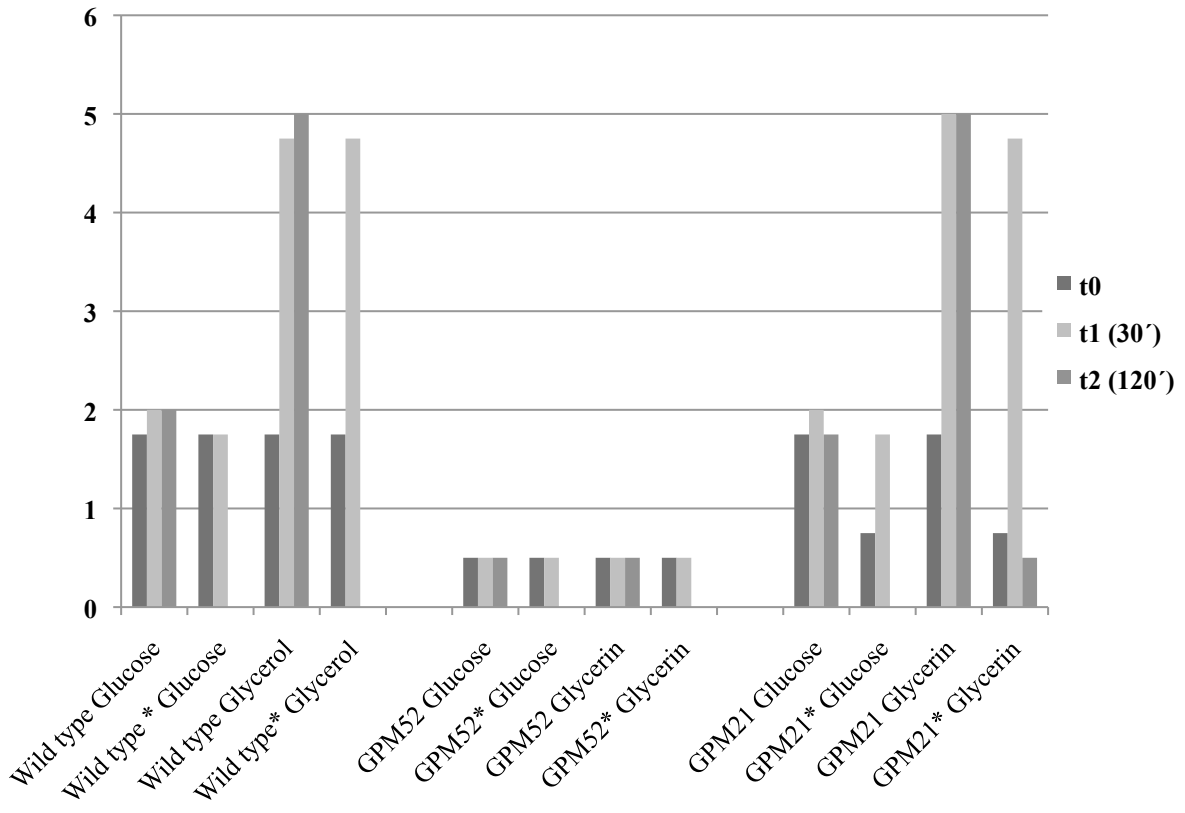


Fig. 3.27: H_2O_2 production of *M. pneumoniae* strains

Production of H_2O_2 by *M. pneumoniae* wild type, GPM52 and GPM21 in the presence of glucose or glycerol. H_2O_2 was measured at three time points (0, 30 min and 120 min). "*" indicates the addition of serine hydroxamate. H_2O_2 concentrations are indicated in [µg/ml].

As described previously and as can be seen in Fig. 3.26, cytotoxicity of *M. pneumoniae* toward human cell lines mainly results from excreted H_2O_2 originating from glycerol metabolism (Hames *et al.*, 2009). Since no differences in cytotoxicity were observed between *M. pneumoniae* wild type and GPM21, the ability of both strains to produce H_2O_2 was studied

by using the Merckoquant peroxide test (Merck, Darmstadt, Germany). *M. pneumoniae* wild type and GPM21 produced equal amounts of hydrogen peroxide using glucose and glycerol as carbon sources. In both strains, H_2O_2 production increased in the presence of glycerol. Addition of serine hydroxamate (1 mg/ml final concentration) to induce (p)ppGpp formation lead to a decrease of H_2O_2 in all culture supernatants. If this decrease is due to inhibited protein synthesis or due to reduction of H_2O_2 catalysed by serine hydroxamate is unknown. Strain GPM52 was used as a control and did only exhibit marginal H_2O_2 production as reported previously in all experiments (Low and Zimkus, 1973; Hames *et al.*, 2009). In summary, no differences in H_2O_2 production were observed between the wild type and GPM21, suggesting no involvement of Mpn397/RelA or (p)ppGpp in the regulation of glycerol metabolism or cytotoxicity.

3.5.7 Microarray analysis of *M. pneumonie* GPM21

(p)ppGpp is involved transcription regulation in all bacteria studied so far. To study differences in transcription of *M. pneumoniae* wild type and strain GPM21 in response to nutrient starvation, microarray analyses were performed.

Transcription pattern of GPM21 under standard conditions

To ensure that disruption of *mpn397/relA* by the transposon or expression of the *aac-ahpD* resistance cassette coded on the transposon does not affect transcription in GPM21, microarray analyses of cultures grown for 6 h were performed. Previous experiments showed that cultures of different strains could be compared best within the first 24 h. No differences in transcription are observed between the wild type and GPM21. This is in good agreement with the finding that no (p)ppGpp was detectable in cultures untreated with serine hydroxamate: nutrient supply is high and Mpn397/RelA should therefore be inactive (chapter 3.5.2). Only transcription of *mpn397/relA* occurred up-regulated in strain GPM21 in these experiments, probably due to the fact that the probe detecting *mpn397/relA* transcripts is located downstream of the transposon insertion and affected by read-through originating from the *aac-ahpD* promoter (Fig. 3.28). This effect is also visible in other strains as will be shown in the next chapters.

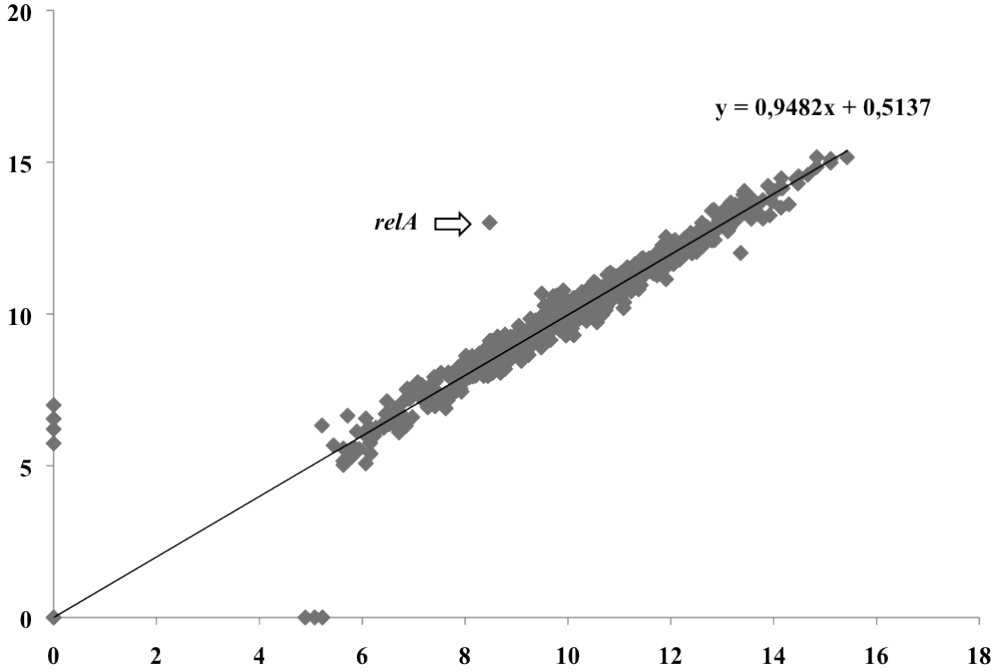


Fig. 3.28: Scatter plot of microarray signals from *M. pneumoniae* wild type and GPM21.
Only transcription of *mpn397/relA* occurs up-regulated in GPM21 under these conditions due to polar effects of the *aac-ahpD* promoter located inside the transposon.

Transcriptional response to a glucose downshift

Glucose starvation was shown to induce the stringent response in *B. subtilis* (Bernhardt *et al.*, 2003; Koburger *et al.*, 2005). To study the glucose starvation response and probable influence of Mpn397/RelA in *M. pneumoniae*, a defined medium would be required. Since no defined medium is available, the transcriptional response to a glucose downshift was analysed instead. *M. pneumoniae* wild type and GPM21 were grown for 48 h in Hayflick medium containing glucose (t_0, see Fig. 3.29). Then, the medium was discarded and cells covered with 50 ml Hayflick without any carbon source, pre-warmed at 37°C. RNA samples were taken after 2 h, 4 h and 6 h and the transcriptome compared with that of untreated cells at time point t_0 within each strain. However, this experimental design has the drawback that two transcriptional responses are observed at the same time: first, transcriptional responses towards downshift of glucose availability and second transcriptional regulation in response to fresh medium. Therefore it is unclear which regulations are influenced by which stimulatory condition and require additional experimental validation.

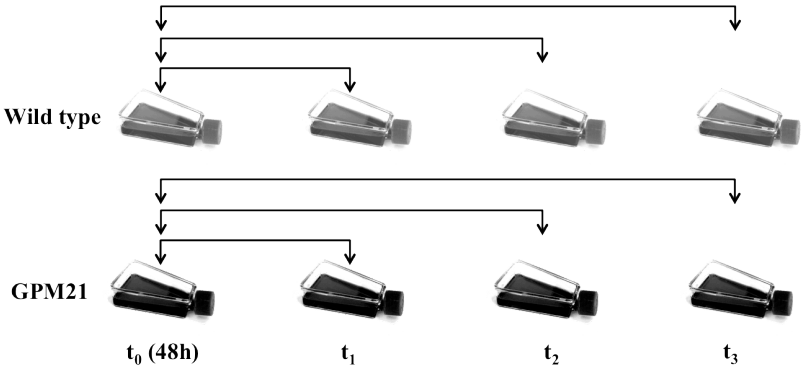


Fig. 3.29: Schematic overview about the glucose downshift experiment.
Transcriptional responses in reference to t_0 were analyzed within each strain.

Only minor differences in transcription were observed between the wild type and GPM21. Because the majority of those differences have fold changes lower than 1.0 and/or pValues >0.5 they are rather thought to be artificial and physiologically irrelevant than strain specific responses. Instead, both *M. pneumoniae* wild type and GPM21 showed almost similar transcriptional responses towards glucose downshift. Both strains showed a down-regulation of transcription of the ATPase operon (ORFs *mpn605* to *mpn596*). In addition, an operon harbouring genes involved in lipid metabolism (ORFs *mpn297* to *mpn302*), a cluster of hypothetical genes (ORFs *mpn459* to *mpn457*) and the *oppB* operon described in chapter 3.3.3 were found up-regulated in both strains after 4 h and 6 h. Interestingly, only proteases *lon* and *clpB* of the HrcA regulon were up-regulated in both strains, too, suggesting a separated mode of regulation in contrast to other genes of this regulon. Finally, the *thyA* operon (ORFs *mpn320* to *mpn324*) harbouring genes involved in nucleotide and vitamin metabolism was down-regulated in both strains. However, if these transcriptional changes are due to the adaptation to fresh medium or to a reduced availability of consumable carbon sources requires additional experiments. Even though an involvement of Mpn397/RelA and (p)ppGpp can be excluded, the mechanisms behind the transcription regulations observed remain unknown.

Transcriptional response towards amino acid starvation

Whereas it is unclear if *M. pneumoniae* synthesizes (p)ppGpp in response to glucose starvation, its ability to synthesize ppGpp in response to amino acid starvation by treatment with serine hydroxamate was shown in chapter 3.5.2. To study the influence of ppGpp on *M. pneumoniae* transcription, an experimental design similar to that described in Fig. 3.29 was chosen: *M. pneumoniae* wild type and GPM21 were grown for 48 h. 25 ml of medium

supernatant were taken from growing cultures und used to dissolve serine hydroxamate or norvaline to a final concentration of 25 mM and 10 mg/ml, respectively. Norvaline is a non-proteinogenic amino acid that competes with leucine in being loaded to its respective tRNA, thereby mimicking amino acid (i.e. leucine) starvation. It has been used previously to study the stringent response in *B. subtilis* (Belitskiĭ and Shakulov, 1980; Riedel *et al.*, 1987; Eymann *et al.*, 2002). After serine hydroxamate or norvaline were dissolved, the residual medium of *M. pneumoniae* strains was discarded and replaced by the same medium containing one of the chemical compounds. After treatment for 15 min (t_1) and 45 min (t_2), cells were lysed and RNA prepared for microarray analysis. Transcription signals were then compared with those from untreated cultures at t_0, analogue to the description in Fig. 3.29. This experimental design allows studying the effect of serine hydroxamate or norvaline without having additional environmental changes such as fresh medium, as described above.

Transcriptional responses of both *M. pneumoniae* wild type and GPM21 in response to norvaline treatment differed strongly. However, they also differed between the replicates of each strain, and thus lead to high pValues for the calculated fold changes. The reason for this is unknown since all cultures were treated in the same manner. Therefore the data of this experiment is not further regarded in this work.

As described for the response towards norvaline treatment, the transcriptional responses of both *M. pneumoniae* strains differed strongly when treated with serine hydroxamate, but were more reproducible in this experiment. However, differences in transcription do not resemble any described phenotype of *relA* mutants in other bacteria. Major differences in transcription were noticed in strain GPM21 originally expected to be unresponsive toward amino acid starvation, whereas only minimal changes occurred in the wild type. For example, a cluster of putative cytadherence genes (ORFs *mpn364* to *mpn370*) was up regulated in GPM21 but not in the wild type, as well as a cluster of conserved hypothetical proteins containing S7 peptidase domains (ORFs *mpn182* to *mpn179*) and the *mpn320/thyA* operon. To test whether these differences between GPM21 and the wild type are due to specific responses towards amino acid starvation or resulting from different growth states of the two strains, transcription signals of t_0 were compared. Analysis of this data suggests that both strains were indeed in different growth states when the experiment was performed, making it impossible to differentiate between growth state specific transcription patterns and strain specific responses towards serine hydroxamate treatment.

Despite these differences, also similar transcription responses were observed in both strains. Both strains down-regulated transcription of a cluster of genes encoding ribosome associated

GTPases Mpn475/EngA and Mpn481/EngB and valine tRNA synthetase Mpn480 (ORFs *mpn482* to *mpn475*). Interestingly, transcription of genes encoding other tRNA synthetases such as Mpn005/SerS, Mpn023/MetS or Mpn402/ProS was also found to be down-regulated in both strains. In addition, transcription of the *mpn516/rpoB* operon was up-regulated in both strains, whereas other genes up-stream of the Mpn516/RpoB promoter were down-regulated.
In summary, no specific transcriptional phenotype could be identified related to Mpn397/RelA or (p)ppGpp. On the other hand these data suggest that *M. pneumoniae* indeed carries out specific responses both towards glucose and amino acid starvation, independently from (p)ppGpp. However, the mechanisms behind these regulation events and their physiological relevance require additional experimental validation.

3.6 Experiments aiming to characterize the role of HPr phosphorylation

Even though the mechanisms of phosphorylation of phosphocarrier protein HPr have been studied extensively in *M. pneumoniae*, the function of HPr(Ser-P) is still unknown in this organism. However, the availability of strains GPM51 (*hprK::Tn*) and GPM68 (*prpC::Tn*) offers the possibility of experimental designs that allow screening for antagonistic phenotypes within these two strains.

3.6.1 Microarray analysis of GPM51 and GPM68

To test whether the phosphorylation state of HPr affects transcription, microarrays have been performed with *M. pneumoniae* strains GPM51 and GPM68 and were compared with transcription signals of the wild type. For this experiment, growth curves were performed and RNA samples taken at time points 6/24/48/72/96 h to overcome the problem that the growth state of *M. pneumoniae* cultures cannot be monitored by measuring the optical density (see discussion). Transcription of genes was regarded to be directly affected by the gene knock-out if the regulatory event occurred along the whole growth curve. Because RNA extraction at time points later then 48 h was not successful for all strains, only the first three time points were used for this study.
In GPM51, only *mpn223/hprK* itself was found to be significantly down-regulated constantly along the growth curve, a polar phenotype due to the transposon insertion as already described for GPM21. In GPM68, genes *mpn245/def* and *mpn246/gmk* upstream of the transposon insertion are affected in addition to *mpn247/prpC* itself. Transcription read-

through of (+) DNA strand by the *aac-ahpD* promoter leads to a significant down-regulation of *mpn246/gmk* and an up-regulation of *mpn245/def* transcription, due to their location on opposite DNA strands. However, no antagonistic transcription patterns were observed in GPM51 and GPM68, and no constant changes in transcription of single genes or operons were noticed in both strains beside the described polar effects of the transposon insertion. This finding suggests that neither the phosphorylation state of HPr nor that of other targets described for Mpn247/PrpC are involved in transcription regulation in *M. pneumoniae*.

3.6.2 Real-time monitoring of glucose consumption by GPM51 and GPM68

Beside of being involved in transcription regulation, the phosphorylation state of HPr was also shown to directly affect the uptake of PTS sugars in *B. subtilis*. HPr(Ser46-P) inhibits uptake of PTS sugars due to a conformational change of the protein the prevents participation in phosphotransfer to the EII complex in the membrane (Reizer *et al.*, 1989).

As shown in chapter 3.5.5, glucose consumption and acidification of the medium by lactate and acetate, the end products of glycolysis in *M. pneumoniae*, directly correlate with each other. To study if HPr phosphorylation at Ser46 affects the uptake of glucose in *M. pneumoniae*, time dependent acidification of the medium by *M. pneumoniae* wild type, GPM51 and GPM68 was monitored. To achieve this, equal cell amounts of *M. pneumoniae* strains were seeded in 96-well microtiter plates containing minimal medium with defined concentrations of glucose and incubated at 37°C for 120 h. Acidification was monitored by hourly measuring of the absorbance at 430 nm and 560 nm to determine the colour change of pH indicator phenol red from red to yellow, as described previously (Yus *et al.*, 2009).

As can be seen in Fig. 3.30, no acidification of the medium was observed in wells without glucose, which served as negative controls. Acidification of the medium by the wild type stopped slowly after about 40 h and stayed constant until the end of the experiment. In contrast, acidification by GPM68 stopped about 10 h earlier and at a higher pH compared to the wild type, indicating an earlier stop of glucose consumption. The opposite effect was observed for GMP51. Acidification by this strain stopped abruptly after about 60 h at a much lower pH compared to the wild type. Thus, this data suggests that uptake or utilisation of glucose by *M. pneumoniae* is regulated in a pH-dependent manner, and that this regulation is mediated by the phosphorylation state of HPr. This hypothesis is in good agreement with observations from HPr in *B. subtilis* showing that HPr(Ser46-P) cannot participate on phosphate transfer to EII of the PTS (Reizer *et al.*, 1989): Whereas *M. pneumoniae* wild type

stops consumption of glucose at a certain pH, strain GPM51 lacking HPrK consumes all glucose available, leading to the abrupt stop of acidification, as observed. In contrast, strain GPM68 unable to dephosphorylate HPr(Ser46-P) is more sensitive to a lowered pH, leading to an early stop of glucose consumption. Hence, glucose uptake in *M. pneumoniae* seems to be regulated by the phosphorylation state of HPr, which itself is modulated by an unknown feedback mechanism. If the feedback signal triggering HPr (de-)phosphorylation indeed is a lowered pH or accumulation of acidic end products of glycolysis, lactate or acetate, requires additional experimental validation.

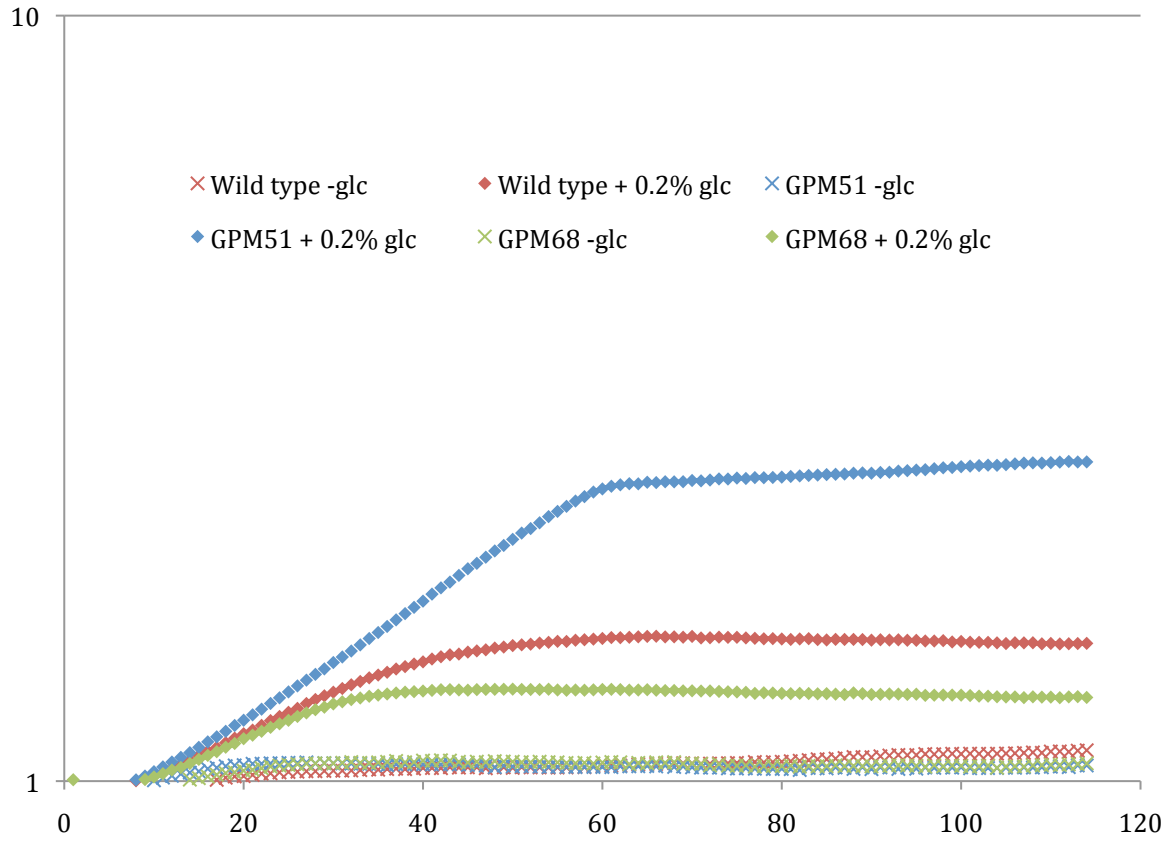


Fig. 3.30: Real-time acidification of defined medium by *M. pneumoniae* strains

X-axis: time [h]; Y-axis: [log] acidification as described in Yus *et al.*, 2009. Concentration of glucose is (w/v).

3.7 Experiments aiming to characterize the role of Mpn241/WhiA

Even though the putative transcription regulator Mpn241/WhiA is conserved in all Gram-positive bacteria, nothing is known about its function or its possible targets. To study its putative role in transcription regulation in *M. pneumoniae*, microarrays with GPM22 (*mpn241/whiA::Tn*) were performed and transcription signals compared with the wild type.

For this purpose, the same experimental setup and the same criteria for directly regulated genes were used as for strains GPM51 and GPM68 (see chapter 3.6.1 and discussion).

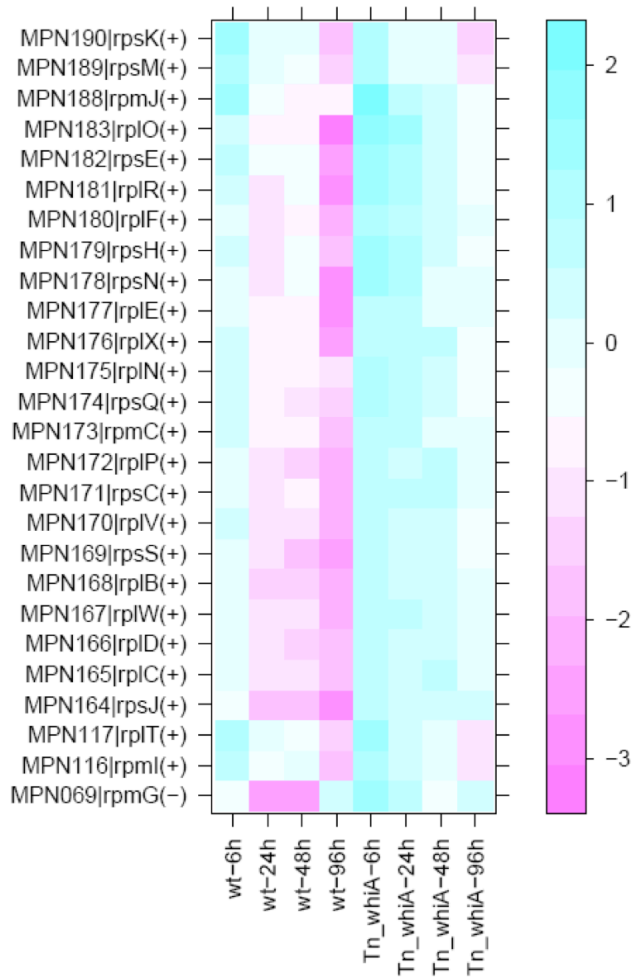


Fig 3.31: Heat map display of transcription signals from the *mpn164/rpsJ* operon

Transcription of the operon (*mpn*164 to *mpn188*) decreases along the growth curve in wild type cells, whereas it stays constantly high in GPM22. Genes *mpn189* and *mpn190* are not part of the operon and exhibit similar transcription patterns in both strains. Down-regulation of transcription of this operon along the growth curve has been described previously (Güell *et al.*, 2009). wt: wild type; Tn_whiA: GPM22. Time points are indicated.

Transcriptome data for time points 6 h, 24 h, 48 h and 96 h were available for both GPM22 and the wild type. Polar effects on neighbouring genes caused by the transposon insertion lead to an up-regulation of transcription of *mpn241/whiA* itself and a remote up-regulation of downstream genes *mpn242/secG* and *mpn243/rnr*. In addition, a constitutive up-regulation of transcription of the *rpsJ* operon was observed in GPM22, whereas transcription of this operon decreased along the growth curve in the wild type. This operon has a size of about 13 kbp and consists of 25 genes (ORFs *mpn164/rpsJ* to *mpn188/rpmJ*), 21 of which encode ribosomal proteins. Transcription of other ribosomal genes was not affected in the mutant. Because up-regulation of this operon occurs along the whole growth curve it meets the criterion to be

directly regulated by Mpn241/WhiA. However, the exact mechanism of regulation and the role of Mpn241/WhiA in regulating this operon require additional experiments.

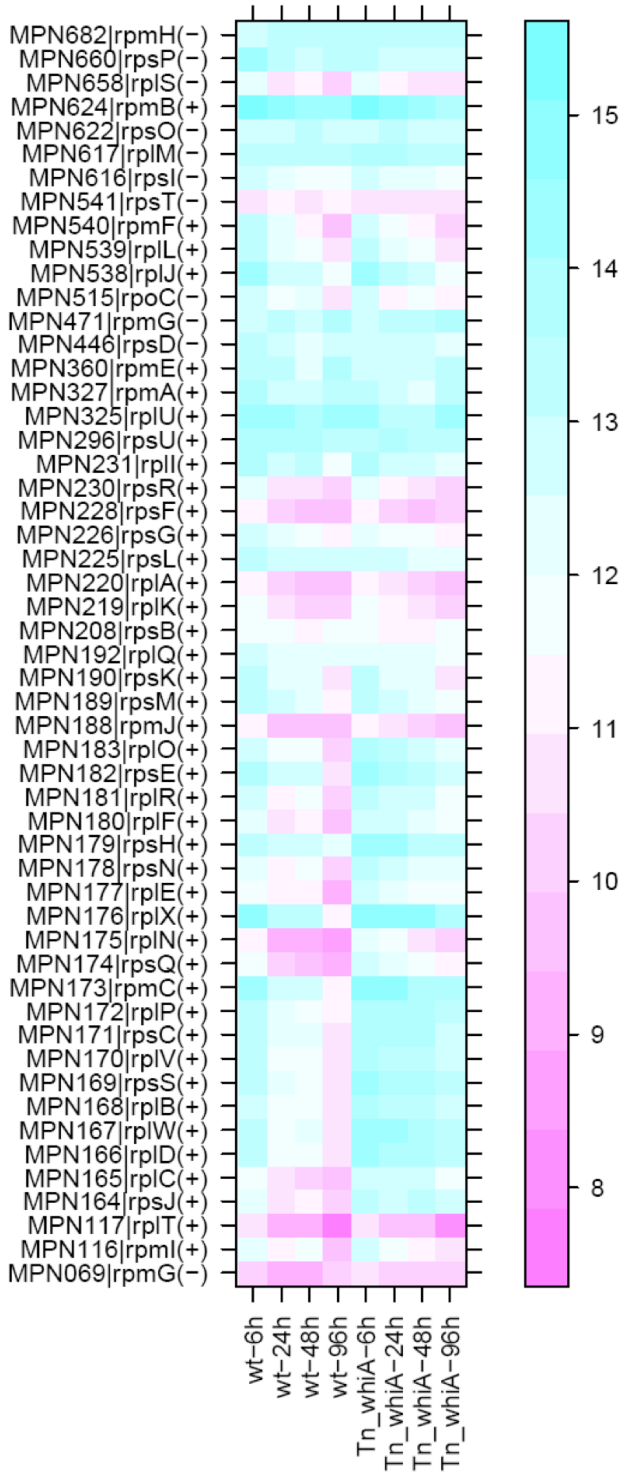


Fig 3.32: Heat map display of transcription signals from genes encoding ribosomal proteins

Only genes encoding ribosomal proteins located in the *rpsJ* operon are affected in transcription by the *mpn241/whiA* disruption in strain GPM22.

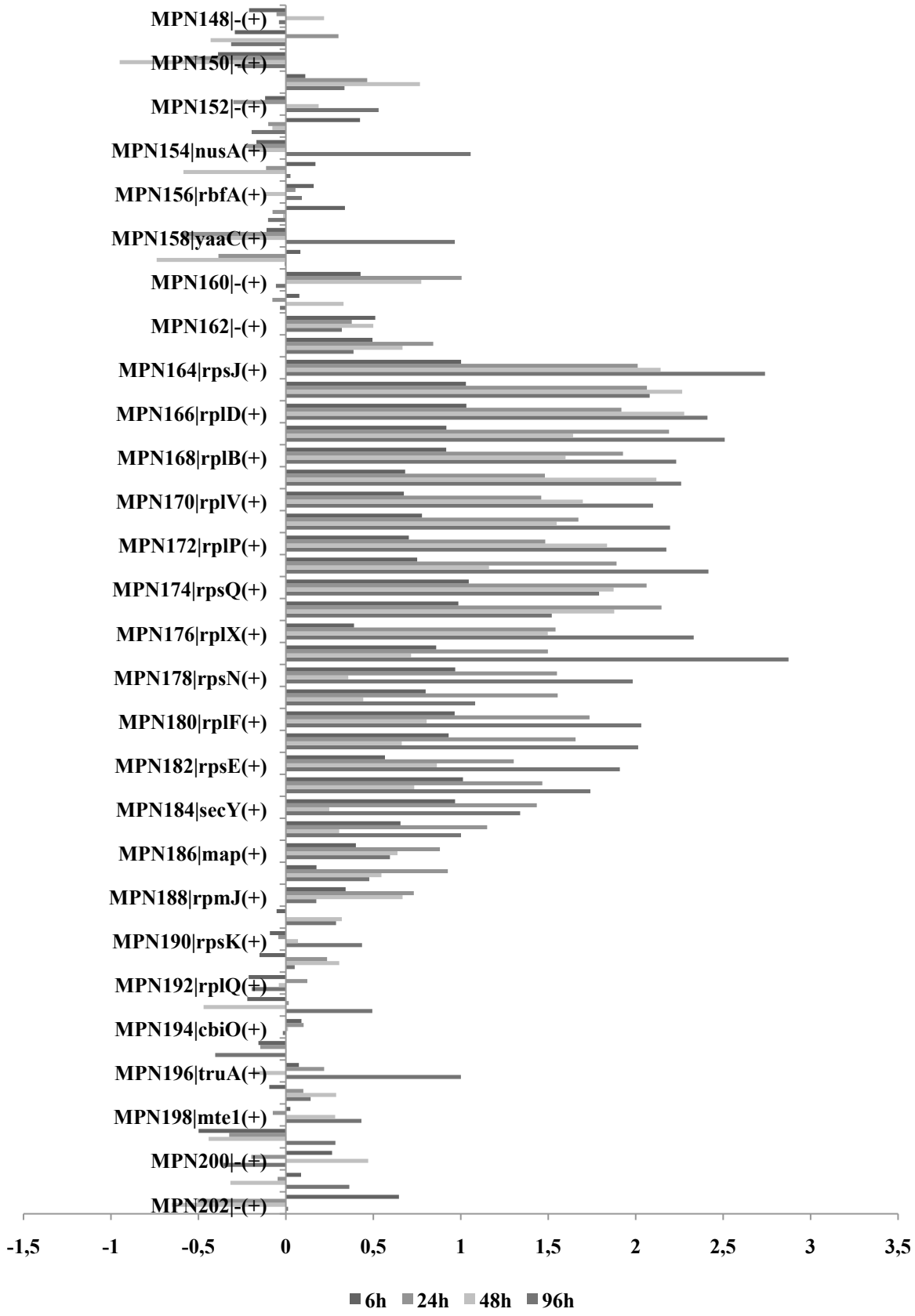


Fig. 3.33: Fold changes of transcription of the *rpsJ* operon

A specific up-regulation of the *rpsJ* operon along the growth curve is observed in GPM21 in comparison to the fold changes (X-axis) of surrounding genes.

4. Discussion

4.1 Last men standing: essentiality of transcription regulators in *M. pneumoniae*

M. pneumoniae lives as a parasite in a rather constant and nutrient rich environment, the human lung. It was proposed previously that this close adaption to its host is not only reflected by its reduced genome, but also by a reduced ability to regulate transcription: only a handful of genes putatively involved in transcription regulation are encoded in its genome (Ochman and Davalos, 2006). Tab. 4.1 below supports this hypothesis. Obligate pathogenic bacteria highly adapted to eukaryotic hosts such as *Phytoplasma*, *Mycoplasma* or *Chlamydia* tend to have smaller genomes and a lower number of transcription regulators compared to ubiquitous, facultative pathogenic bacteria like *Bacillus* or *Pseudomonas*.

Genome	Genes	Predicted TF	Average genes per TF
(Human mitochondria)	(37)	(0)	(0)
Onion Yellows Phytoplasma OY-M	754	2	377
Mycoplasma pneumoniae	689	4	172.3
Chlamydia trachomatis D/UW-3/CX	895	4	223.8
Treponema pallidum ssp. *pallidum* Nichols	1036	6	172.7
Helicobacter pylori 26695	1576	8	197
Campylobacter jejuni RM1221	1838	24	76.6
Neisseria meningitidis MC58	2063	49	42.1
Streptococcus pneumoniae G54	2115	95	22.3
Staphylococcus aureus ssp. *aureus* N315	2588	121	21.4
Bacillus anthracis Ames	5311	298	17.8
Pseudomonas aeruginosa PA7	6286	436	14.4

Tab. 4.1: Transcription regulators in pathogenic bacteria.

Numbers of genes and predicted transcription factors (TF) in selected pathogenic bacteria, sorted by their number of genes. Genome reduction and loss of regulatory potential are correlated in obligate pathogenic bacteria whereas opposite trends are visible in facultative pathogens (top to bottom). The data were taken from www.transcriptionfactor.org (Wilson *et al.*, 2008).

Thus, adaption to a eukaryotic host providing nutrients and offering a stable environment allows bacteria to reduce their genome and decreases the need to regulate gene expression. This results in a degenerative evolution of the genome with a simultaneous increase of

dependency on the host, as proposed for *Mycoplasma* (Woese *et al.*, 1979). This phenomenon raises two important questions: What is the minimal number of genes required for autonomous growth, and what is the minimal requirement of regulation for these genes?

Whereas answers to the first question were given in previous studies, only little is known about regulatory events in minimal organisms, such as *M. pneumoniae*. In this study, a transposon insertion library was screened for viable disruption mutants of genes putatively involved in transcription regulation. Only two genes were found to be non-essential, i.e. *mpn397/relA* and *mpn241/whiA*. The finding of several essential transcription regulators is surprising since only very few cases in bacteria have been described yet, among them the essential WalK/R two-component system conserved in gram positive bacteria with the exception of the *Mollicutes* (Fabret and Hoch, 1998). However, this also raises the question why these regulators are essential, whereas their orthologues in other bacteria are not. For example, *spx* mutants have been described in *B. subtilis*, *S. aureus* and *S. mutants* while the orthologue Mpn266/Spx is essential in *M. pneumoniae* (Nakano *et al.*, 2001; Pamp *et al.*, 2006; Kajfasz *et al.*, 2009). Three general possibilities could explain the essentiality of Mpn266/Spx and the other transcription regulators: (I) The transcription regulator is constantly required and therefore indispensable for cellular processes, (II) it is required under the laboratory growth conditions and therefore dispensable under certain, unknown conditions and (III) it is essential because disruption of the gene leads to polar effects on the neighbouring genes that affect viability of the cell.

In the case of *mpn266/spx*, all three explanations could account for its essentiality. In contrast to its orthologues in other bacteria, expression of *mpn266/spx* is not regulated in *M. pneumoniae* and it constantly interacts with the core RNA polymerase *in vivo*, as concluded from co-purification experiments (Güell *et al.*, 2009; Kühner *et al.*, 2009). Therefore the author of this thesis proposed that Mpn266/Spx might have become an integral part of the *M. pneumoniae* RNA polymerase that helps to overcome the weakly defined promoters by allowing it to bind to additional, *cis*-acting promoter elements. However, no consensus sequence up- or downstream of the putative -35 region of promoters was detected that could serve as additional or alternative promoter element, neither in two independent studies focussing on transcription in *M. pneumoniae* nor in this work, making it difficult to verify this hypothesis (Weiner *et al.*, 2000; Güell *et al.*, 2009). On the other hand, Mpn266/Spx might be only essential under laboratory growth conditions, due to oxidative stress. Two independent sources of H_2O_2 have been described in *M. pneumoniae*, with the majority resulting from glycerol metabolism (Low and Zimkus, 1973; Hames *et al.*, 2009).

H_2O_2 can lead to the formation of oxygen radicals by the Fenton reaction, and thus to oxidative stress. Spx orthologes are transcriptional activators responding to thiol specific oxidative stress, and the responding CxxC motif at the N-terminus of Spx proteins is also conserved in Mpn266/Spx. Therefore it is tempting to speculate that this protein fulfils a similar role in *M. pneumoniae*. Because the medium used to grow *M. pneumoniae* contains 20% horse serum and hence a certain amount of glycerol or glycerol-3-phosphate, a permanent accumulation of H_2O_2 accompanied by an increasing acidification of the medium due to glycolytic activity during growth is very likely. Both H_2O_2 and acidification are prerequisites for the Fenton reaction and could therefore be the cause of permanent oxidative stress in *M. pneumoniae* during growth in Hayflick medium, making Mpn266/Spx indispensible under this condition. Finally, *mpn266/spx* might be essential due to the essentiality of its neighbouring genes. *mpn266/spx* is the first gene of a five-gene operon, incoding a putative inorganic polyphosphate/ATP-NAD kinase (*mpn267*) and the orthologue of RNaseY (*mpn269*) described in *B. subtilis* (Commichau *et al.*, 2009; Shahbabian *et al.*, 2009). Besides *mpn266/spx*, two genes of this operon are essential as was shown in previous studies, and at least RNaseY is essential in other bacteria as well (Hutchison *et al.*, 1999; Glass *et al.*, 2006; Kobayashi *et al.* 2003). Therefore, disruption of Mpn266/Spx by a transposon could prevent transcription of neighbouring essential genes or interfere with transcription regulation of the whole operon, thereby becoming essential due to polar effects instead of a required function of the protein itself.

In the case of Mpn329/Fur, the essentiality presumably results from the fact that it is required to regulate one or several target genes whose uncontrolled expression would be cytotoxic for the cell. None of the genes downstream of *mpn329/fur* is essential, eliminating the possibility of polar effects by transposon insertion as described for *mpn266/spx*. Furthermore, all members of the Fur-family in Gram-positive bacteria described so far are repressors responding to metal homeostasis or, in the case of PerR, to peroxide stress. To date, no bacterial transcription repressor has been reported that becomes essential under certain conditions. Assuming that Mpn329/Fur also acts as a repressor in *M. pneumoniae*, it is therefore more likely that this protein is required to prevent over-expression of harmful target genes. Different approaches have been used to identify target genes of Mpn329/Fur in this work. Several putative targets have been identified fulfilling the predicted phenotype of Fur-family proteins, i.e. up-regulation during starvation on the cofactor Fe^{2+} required to obtain DNA binding ability. However, none of the putative target genes harbours a DNA binding-motif in its promoter region resembling that of Fur-family proteins in other bacteria, making it

difficult to proof a direct regulatory effect by Mpn329/Fur on these genes (as will be discussed in chapter 4.4 below). Because no target could be identified, it is still unclear if Mpn329/Fur belongs to the Fur, PerR or Zur subfamily of Fur proteins. In other bacteria, Fur regulates the expression of genes involved in synthesis, secretion and uptake systems of siderophores in response to depleting Fe^{2+} to maintain iron homeostasis. However, human pathogenic bacteria have to overcome the problem that no free Fe^{2+} is available since it is bound to specialized chelator proteins like lactoferrin or transferrin, a passive defence against microbial colonization. Many bacteria therefore have adapted to their host by developing specialized transporters or binding proteins to utilize lactoferrin or transferrin as sources of Fe^{2+}, like the human pathogen *N. meningitidis* (Mickelsen and Sparling, 1982; Schryvers and Morris 1988). In good agreement with its close adaption to its host, *M. pneumoniae* was also shown to specifically bind human lactoferrin, providing evidence for a specialised transport system (Tryon and Baseman, 1987). If such a transport system were present in *M. pneumoniae*, it would likely be under the control of Fur. However, despite the OppB peptide transporter conserved among bacteria, no putative transport system or membrane anchored protein was up-regulated during iron starvation, suggesting that Mpn329/Fur does not belong to the Fur subgroup. As mentioned previously and in good agreement with these findings, Mpn329/Fur shows a higher degree of homology with PerR proteins from several *Firmicutes* than with Fur. The presence of a peroxide responsive transcription regulator makes sense in *M. pneumoniae* because it would allow triggering detoxification from self-produced H_2O_2 with concomitant protection of the redox-state of the cell. However, no enzyme neutralizing H_2O_2 known in other bacteria such as catalase is present in the genome of *M. pneumoniae*. One of the genes up-regulated in response to iron starvation is *mpn394/nox*, a predicted NADH oxidase. Interestingly, PFAM and BLAST analyses show that these proteins share domain homologies with several redox-acting enzymes, among them NADH dependent peroxidases. However, enzymatic activity of Mpn394/Nox to oxidate NADH was shown previously (C. Hames, doctoral thesis). Moreover, no conserved DNA motif was found in the promoter region of this gene (see chapter 4.4 below). Even though a participation of Mpn394/Nox in H_2O_2 detoxification cannot be excluded, a direct regulation by Mpn329/Fur is very unlikely. Assuming that Mpn329/Fur indeed is a PerR orthologue in *M. pneumoniae*, one reason why no putative target could be identified by the experiments performed might be permanent oxidative stress by H_2O_2 formation under laboratory growth conditions, as already discussed for Mpn266/Spx. H_2O_2 originating from glycerol or glycerol-3-phosphate degradation could lead to a permanent induction of Mpn329/Fur regulated genes,

making a further induction by iron depletion marginal or impossible. To proof this hypothesis, a serum-free medium would be required containing minimal amounts of sources for glycerol or glycerol-3-phosphate. Until this, alternative methods to characterize and to identify targets of Mpn329/Fur have to be developed.

Even though almost known for 20 years, essentiality of HrcA orthologues in other bacteria than *M. pneumoniae* has not been reported to date. Essentiality of Mpn124/HrcA due to polar effects can be excluded due to its monocistronic transcription, leaving cytotoxic effects of its target genes the only logical reason to explain this phenotype. Mpn124/HrcA controls the transcription of chaperones and proteases in *M. pneumoniae* and other bacteria. However, in contrast to other bacteria, only three putative proteases are present in the genome of *M. pneumoniae*, two of which are under control of HrcA: Mpn332/Lon and Mpn531/ClpB (Chang *et al.*, 2008). Both proteases have been characterized previously (Gur and Sauer, 2008; Ge and Krazai, 2009; Kannan *et al.*, 2008). The third protease, Mpn671/FtsH is conserved in most bacteria and involved in the proteolysis of cell division proteins (Schumann, 1999). Hence, deletion of HrcA would lead to an increased expression of the only two proteins putatively involved in protein turnover in *M. pneumoniae*. In *B. subtilis*, more then 30 putative proteases can be found (http://subtiwiki.uni-goettingen.de/) that could help to degrade HrcA-dependent proteases like Lon or ClpB, thereby compensating HrcA deletion in this organism. Thus, uncontrolled expression of proteases might be cytotoxic for *M. pneumoniae* and could explain the requirement of Mpn124/HrcA for viability. The finding that perfect CIRCE elements are only found upstream of *M. pneumoniae mpn332/lon* and *mp531/clpB* and that CIRCE is even missing upstream of *M. genitalium groEL/ES* is supporting this hypothesis (Chang *et al.*, 2008).

mpn239/gntR is located in the middle of a large operon. Its ORF overlaps with those of its neighbouring genes, *mpn238/gatB* and *mpn240/trxB*, both of which are essential. Therefore it is tempting to speculate that essentiality of Mpn239/GntR results from the essentiality of its neighbouring genes. However, essentiality of this regulator might also result from a requirement of tight control of its target genes, either in general or only under laboratory growth conditions used.

To answer the question whether a regulator is essential due to its function or due to its location in the genome, clean deletions or deletions that maintain the transcription start point of downstream genes generated by homologous recombination would be required to circumvent polar effects on the neighbouring genes. If the regulator is essential due to its function, several approaches to identify its targets can be imagined. Replacement with an

inducible expression system coupled with global analysis such as microarray or 2D-gels has been used to identify targets of the essential WalK/R two-component system or RNaseY in *B. subtilis* (Howell et al., 2003; Martin Lehnik, personal communication). Recent publications show that targeted knockout by homologous recombination is working in *M. pneumoniae*, though with very low efficiency, and the development of a tetracycline inducible expression system functional in the *Mollicutes* was reported previously (Breton *et al.*, 2010; Krishnakumar *et al.,* 2010). Thus, the prerequisites for such an experiment are given. Alternatively, this experiment could also be performed in the close relative *M. genitalium* in which homologous recombination works more efficiently. Another method to identify the target of an essential transcription regulator is ChIP-chip, as explained previously. This approach has the advantage that the experiment can be performed with native bacterial cells, thereby preventing construction of artificial strains that might interfere with the experiment.

4.2 Evaluation and interpretation of microarray data from *M. pneumoniae*

Microarray experiments have been used to study transcriptional responses towards certain stimuli or to identify targets of putative transcription regulators in bacteria for quite a while. However, strategies to normalize growth conditions of the bacteria and to interpret the data resulting from such experiments have to be individually defined for each bacterium. In this study, for the first time ever, *M. pneumoniae* strains with transposon insertions in genes putatively involved in transcription regulation have been examined by microarray analysis.

The main difference between *M. pneumoniae* and other bacteria is its adherent growth that prevents normalisation of the growth state of a culture. Whereas the growth state of non-adherent bacteria like *B. subtilis* or *E. coli* can be monitored by measuring the optical density of a culture, this is not possible with *M. pneumoniae*. Furthermore, shaking of cultures of non-adherent bacteria ensures an equal distribution of nutrients and end-products of metabolism. In contrast, local differences in cell density of adherent *M. pneumonia*e cultures lead to differences in nutrient availability or local accumulation of metabolic end-products, and thus to different conditions within a single culture. In consequence, transcription signals of each single culture individualize along the growth curve as can be seen in Fig. 4.1. In this figure, scatter plots of transcription signals of wild type and GPM68 (*prpC::Tn*) from cultures grown for 6 h and 96 h are compared to visualize the decrease of conformity of transcription signals along the growth curve. Thus, all cultures analyzed by microarrays in

this work showed a high degree of conformity within the first 24 h and a decreased conformity within 48 h to 96 h.

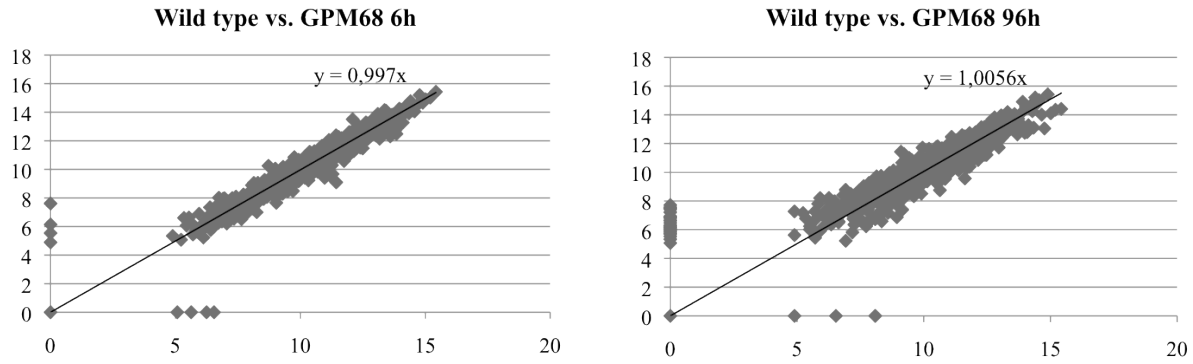


Fig. 4.1: Decrease of conformity of transcription signals along the growth curve

Scatter plots of *M. pneumoniae* wild type and GPM68 transcription signals at time points 6 h and 96 h. The longer different strains grow, the less transcriptional signals correlate due to a lack of culture normalisation.

The aim of this work was to identify genes that are directly or indirectly controlled by the genes knocked out in the mutant strains. To overcome the lack of culture normalisation and to allow differentiating between differences in transcription that are either due to the genetic knockout or resulting from the different growth states of two cultures compared, the following hypothesises have been developed:

I. A gene is under direct control of the knocked out gene or influenced by a certain stimulus (such as iron depletion) if a regulatory effect is visible (in the mutant strain or treated culture) along the whole growth curve.

II. The direction of regulation of the gene, i.e. up- or down-regulation of transcription, has to be constant along the growth curve.

III. If a regulated gene is located within an operon, downstream genes should be affected by the regulatory event.

These hypotheses were made from experiences of previous microarray experiments with *M. pneumoniae* and mainly result from observations of the HrcA regulon: all genes of this regulon are constantly up-regulated along the growth curve at elevated temperatures, and downstream genes are affected by transcription read-through, as also observed in this work (Weiner *et al.*, 2003; Güell *et al.*, 2009). Hence, genes that are regulated in a Fe^{2+} dependent

manner or whose regulation depends on genes *hprK/ prpC/ relA/ whiA* would be constantly up- or down-regulated in cultures grown in the presence of the iron chelator 2,2´-dipyridyl or cultures of the corresponding mutant strains, respectively. As will be discussed below, the transcription pattern of GPM22 (*mpn241*) matches these criteria. A constant up-regulation of transcription of the 25 genes long *mpn164/rpsJ* operon is visible in this strain when compared to the wild type, thereby serving as a proof-of-principle for the hypotheses discussed above. In contrast, with the exception of polar effects by the transposon insertion, no such behaviour was observed for any gene in the mutant strains GPM21 (*relA*), GPM51 (*hprK*) or GPM68 (*prpC*), suggesting that these genes are neither directly nor indirectly involved in transcription regulation under the conditions tested.

Compared to other bacteria that have been investigated by microarray analysis, induction factors by mutation of the putative transcription regulator Mpn241/WhiA are somewhat low. Whereas fold changes of 30 and higher are observed on *E. coli* or *B. subtilis*, the highest induction of the *mpn164/rpsJ* operon observed is fold changes of about 3. However, several reasons could explain this phenomenon. First, the use of different microarray platforms that deliver different signal intensities and thus different fold change calculations. Second, massive data normalisation is required to compare cultures of different strains due to the fact that growth of the cultures themselves cannot be normalized, as discussed above. Finally, a comparatively low abundance of RNA polymerase has been reported for *M. pneumoniae*, leading to extremely low levels of mRNA and hence to a lower induction of transcription (Weiner *et al.*, 2003; Kühner *et al.*, 2009).

4.3 Sequence conservation and *cis*-acting regulatory elements in *M. pneumoniae*

A general problem in working on transcription in *M. pneumoniae* is the fact that DNA sequences involved in transcription initiation or regulation are only weakly conserved. As reported previously, promoter sequences lack a consensus -35 region and show a high variability of the central bases of the -10 region (Weiner *et al.*, 2000; Halbedel *et al.*, 2007; Güell *et al.*, 2009). Interestingly, Mpn352/SigA shows an unusual amino acid composition in domain 4.2 responsible for recognition and binding to -35 regions of promoters, whereas domain 2.4 responsible for recognition and binding to -10 regions is highly conserved (Fig. 4.2; Gruber and Groß, 2003). This observation results in a “hen or egg” question: are weakly conserved promoters a consequence of mutations in conserved domains of the sigma factor, or did the loss of conservation in region 4.2 result from a high sequence variability of *M.*

pneumoniae promoters? The answer to this question might be concluded from a recent publication on transcription in the human pathogen *H. pylori* (Sharma *et al.*, 2010). As shown in Tab. 4.1, this bacterium has a rather small genome that harbours only very few putative transcription regulators just like *M. pneumoniae*, and transcription is initiated by only three different sigma factors. Thus, the equipment of *H. pylori* to initiate and regulate transcription somewhat resembles that of *M. pneumoniae*. Interestingly, *H. pylori* promoters also resemble those of *M. pneumoniae*: they only consist of a -10 region with a high sequence conservation of the outer bases, TANNAT. Multiple alignments of regions 2.4 and 4.2 from different housekeeping sigma factors show that domain 4.2 of *H. pylori* SigA has 8 non-conserved amino acids in this domain, 5 of them at the same positions as the mutations in domain 4.2 of SigA from *M. pneumoniae* (Fig. 4.2). In domain 2.4, 2 of 4 deviations from the consensus are located at the same positions in the sigma factor of both bacteria. Thus, even though only distantly related, both bacteria have developed mutations in the same positions of their housekeeping sigma factor, leading to a similar promoter sequence recognized by these proteins.

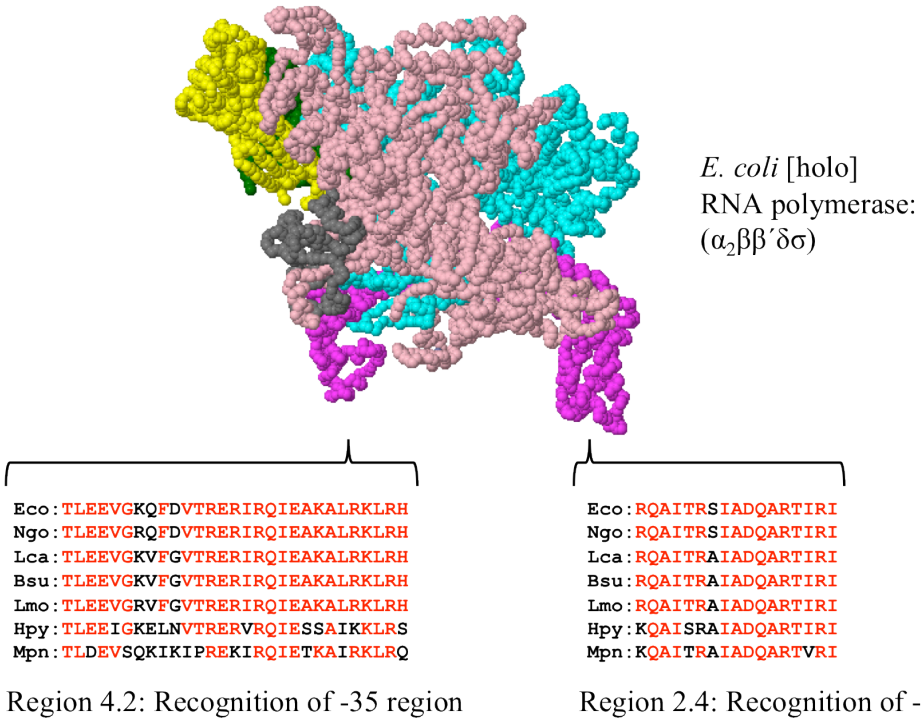


Fig. 4.2: Mutations in the amino acid sequence of conserved domains in housekeeping sigma factors

Protein structure of the *E. coli* [holo] RNA polymerase consisting of α (green and yellow), β (blue), β´ (rose), δ (grey) and σ (pink). Below: multiple alignments of regions 2.4 and 4.2 of the house-keeping sigma factor from *Escherichia coli* (Eco), *Neisseria gonorrhoe* (Ngo), *Lactococcus casei* (Lca), *Bacillus subtilis* (Bsu), *Listeria monocytogenes* (Lmo), *Helicobacter pylori* (Hpy) and *Mycoplasma pneumoniae* (Mpn). Deviations in the same amino acid residues might be responsible for similar promoter requirements of *M. pneumoniae* and *H. pylori*.

Both *M. pneumoniae* and *H. pylori* have in common that transcription is initiated with only few sigma factors. Therefore they could tolerate mutations in the amino acid composition of their sigma factors that lead to a loss of function in recognizing a defined -35 promoter sequence and, in consequence, resulting in a loss of conserved -35 sequences of promoters. In contrast, bacteria like *B. subtilis* that regulate transcription by recruiting up to 20 different sigma factors (http://subtiwiki.uni-goettingen.de) require highly conserved promoters to prevent transcriptional cross-talk or deregulation of transcription. Hence, promoter conservation depends on the ability of a bacterium to tolerate mutations in its sigma factor that itself seems to correlate with the total number of sigma factors used to initiate transcription. This hypothesis could therefore explain why *B. subtilis* is not able to recognize promoters from *M. pneumoniae*, as was shown in this work.

The same hypothesis discussed above could also explain the weak conservation of *cis*-acting regulatory sequences in *M. pneumoniae*, such as CIRCE. From 5 predicted CIRCE elements, only 2 upstream of protease genes *mpn332/lon* and *mp531/clpB* are perfect inverted repeats without any mismatches. In other bacteria such as *B. subtilis*, genes are often targeted by several different transcription factors and therefore require highly conserved DNA sequences recognized by specific regulators. This is not the case in bacteria possessing only very few transcription factors, therefore these bacteria can tolerate a certain extent of mutations within these regulatory DNA elements. Because a weak conservation of these *cis*-acting elements results in a weak responsiveness toward its regulator, only elements upstream of genes whose regulation is required for viability maintain conserved, as discussed above. This lack of sequence conservation raises difficulties to predict new *cis*-acting elements recognized by the remaining transcription regulators. The fact that all putative transcription regulators present in *M. pneumoniae* can also be found in its close relative *M. genitalium* can help to overcome this problem: since both bacteria possess the same regulators, at least one of their target genes has to be present in both genomes, and thus the *cis* element should be found upstream of these genes.

In this work, several experiments were performed to identify genes that are under control of Mpn329/Fur. Even though several putative target genes could be identified by their phenotype, i.e. up-regulation of transcription under Fe^{2+} depleting conditions, none of these genes neither harboured an inverted repeat resembling Fur- or PerR-boxes upstream of its start codon, nor any other sequence conserved in both *M. pneumoniae* and *M. genitalium*. Therefore it is unlikely that Mpn329/Fur directly controls these genes and the regulation is due to a so far unknown mechanism independent of a conserved *cis*-acting element. On the

other hand, *in silico* sequence analysis using several different databases revealed a conserved sequence upstream of *mpn527* and its orthologe in *M. genitalium* that harbours a complex inverted repeat that strongly resembles Fur- and PerR boxes from *B. subtilis*. *mpn527* encodes for a putative membrane associated oxidoreductase with orthologues in *M. pneumoniae*, *M. genitalium* and *M. gallisepticum*, hence all three *Mycoplasma* species with orthologues of Mpn329/Fur. However, no regulation of *mpn527* was observed in any experiment performed in this work, probably due to permanent oxidative stress as discussed previously. Thus, this work could not provide experimental evidence that Mpn329/Fur regulates this gene. In addition to this putative Fur/PerR-box, *in silico* analysis of intergenic regions from *M. pneumoniae* using the RibEx database (Abreu-Goodger and Merino, 2005) revealed the presence of a novel putative *cis*-acting element upstream of Mpn314/MraZ. MraZ is a protein putatively involved in cell division and widely conserved among bacteria. Interestingly, the discovered motif is found upstream of *mraZ* in all bacteria examined, suggesting a common mechanism of regulation. Expression of *mraZ* by an artificial unregulated promoter leads to cell lysis in *E. coli*, underlining the requirement of regulation of *mraZ* in bacteria. Thus, conservation of this motif might be required to maintain viability of *M. pneumoniae* (Mengin-Lecreulx *et al.*, 1998).

A

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Mpn527:  GTCCATATTAATAAATATTATTATTTAACTACTT-TTCTAGCTAGTAGGCTTTA
Mge521:  GTCCATATCAATAAATATTATTATT-AACTAATAATTCAAGAGTCTTTTAAATA
PerR:         AATTTATAAT-ATTATAAATT
Fur:          AAATGATAAT-ATTATCATTT
```

B

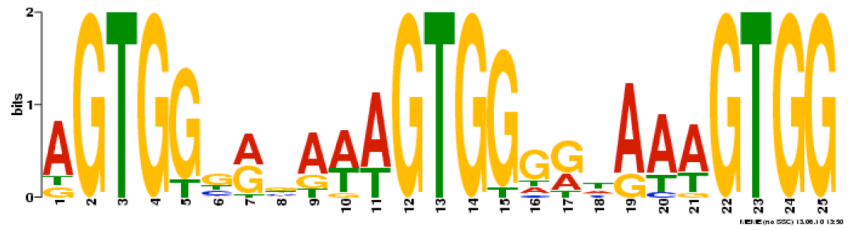

Fig. 4.3: Putative *cis*-acting DNA motifs in *M. pneumoniae*.

A: multiple alignments reveal a conserved sequence within the intergenic region upstream of Mpn527 and Mge521 (red), resembling PerR and Fur boxes from *B. subtilis*. An inverted repeat (9-AA-9) conserved in both *Mycoplasma* species that might be the target sequence of Mpn329/Fur is underlined. B: Novel conserved DNA motif upstream of *mraZ* identified by comparisons of upstream sequences from *M. pneumoniae*, *M. genitalium*, *B. subtilis*, *S. aureus*, *C. glutamaticum*, *E. coli*, *C. tetani*, *T. thermophilis*, *E. faecalis*, *P. aeroginosa*, *M. tuberculosis* and *Y. pestis*. This motif is found in all bacteria possessing *mraZ* but its function is so far unknown.

4.4 *mpn397/relA*: a gene without a function?

The stringent response and the role of (p)ppGpp in transcription regulation in *M. pneumoniae* were one of the main focuses of this work. Even though it could be shown that the mechanisms that lead to (p)ppGpp synthesis are conserved in *M. pneumoniae*, no obvious phenotype of an *mpn397/relA* mutant was observed in a variety of experiments. Moreover, transcriptional responses of the *mpn397/relA* mutant towards amino acid starvation or glucose downshift resembled those of the wild type, suggesting no involvement of (p)ppGpp in transcription regulation in *M. pneumoniae*. In *B. subtilis* and other *Firmicutes*, transcription regulation during the stringent response is a passive effect due to the inhibition of GTP synthesis: (p)ppGpp inhibts the activity of IMP dehydrogenase, a key enzyme of GTP synthesis, leading to reduced levels of intracellular GTP and a lowered synthesis of transcripts that initiate with GTP as start nucleotide (Krásný *et al.*, 2008; Tojo *et al.*, 2010). In *M. pneumoniae*, IMP dehydrogenase is absent and, according to this, preliminary results indicate that intracellular GTP levels were only slightly lowered when ppGpp accumulated in cells treated with serine hydroxamate. Hence, the missing ability to decrease amounts of intracellular GTP could explain the lacking transcription regulation due to ppGpp accumulation. This theory is supported by the fact that *M. pneumoniae* is not able to down-regulate transcription of stable RNA species, neither in response to amino acid starvation nor to glucose starvation (Eva Yus, CRG Barcelona, personal communication). In contrast, down-regulation of rRNA synthesis accompanied with (p)ppGpp accumulation in response to amino acid starvation was reported for *Mycoplasma capricolum* which possesses both RelA and IMP dehydrogenase enzymes (Glaser *et al.*, 1981). Interestingly, the occurrence of IMP dehydrogenase does not correlate with the occurrence of RelA in the *Mollicutes*: analysis of the 27 available *Mollicutes* genomes shows that only 5 members encode an IMP dehydrogenase orthologue, whereas 15 (including those with IMP dehydrogenase) possess a RelA orthlogue. Thus, the ability to induce (p)ppGpp synthesis during nutrient starvation seems to be advantageous for these bacteria, even though it is unlikely to be involved in transcription regulation. This raises the question about alternative functions of (p)ppGpp in these bacteria. Despite of being involved in transcription regulation, (p)ppGpp triggers the activity of a variety of enzymes by interacting with these proteins. For example, (p)ppGpp was shown to interact with the ribosome associated GTPase Obg, translation initiation factor IF-2 and elongation factors Tu, Ts and G, thereby affecting ribosome assembly, translation initiation or peptide elongation in other bacteria (Buglino *et al.*, 2002; Persky *et al.*, 2009; Milon *et al.*, 2006; Roja *et al.*, 1984). Because these proteins are highly conserved among

bacteria and orthologues are also present in the genome of *M. pneumoniae*, it is tempting to speculate that (p)ppGpp can also trigger their activity in this organism. Thus, *M. pneumoniae* could down-regulate protein synthesis without further decreasing the number of ribosomes, whose number is already estimated to only 150 to 300 per cell. This might also explain why no transcription regulation is required. In addition to controlling protein synthesis, (p)ppGpp triggers cell division by inhibiting the enzyme primase to prevent DNA replication (Wang *et al.*, 2007; Maciąg *et al.*, 2010). It was therefore proposed previously that (p)ppGpp synthesis could help *M. pneumoniae* and other *Mollicutes* to persist during nutrient limitation by shutting down metabolic activities and replication. This hypothesis was developed from the finding that *Mycoplasma* species could be recovered from cultures containing high concentrations of puromycin, an antibiotic that targets protein synthesis (Algire *et al.*, 2009). However, repetition of this experiment using *M. pneumoniae* wild type and Mpn397/RelA mutant strain GPM21 showed no advantage of the wild type, and both strains could be recovered even after 96 h incubation with the antibiotic (data not shown). Hence, there is no evidence that Mpn397/RelA helps *M. pneumoniae* to persist nutrient starvation *in vitro*.

Recent findings show that (p)ppGpp is not only involved in triggering regulatory processes that help to overcome nutrient starvation, but also in optimizing protein costs when exposed to changing nutrient availabilities (Shachrai *et al.*, 2010). Protein cost describes the penalty of fitness due to synthesis of unneeded proteins in bacteria: if unused proteins are synthesized, energy in forms of ATP or GTP and amino acids is unnecessarily consumed, leading to disadvantageous effects for the cell. Hence, triggering translation to prevent unnecessary protein synthesis is beneficial for bacteria and may be the reason why RelA is conserved in *M. pneumoniae*. Even though being surrounded by protein in its natural habitat, energy generation by ATP synthesis might be a limiting factor to synthesize proteins, leading to uncharged tRNAs (due to low levels of ATP required for aminoacetylation), and thus to (p)ppGpp synthesis to keep translation rates low. The hypothesis of (p)ppGpp as a general trigger of translation rather then as a trigger of an emergency response toward nutrient starvation rates is supported by the finding that a RelA-independent transcription response towards amino acid starvation or glucose down-shift was observed, as already observed in *S. pyogenes* (Steiner and Malke, 2000).

Finally, the possibility that long-term starvation leads to a decreased pool of intracellular GTP, due to an exhaustive conversion to (p)ppGpp in these bacteria that leads to transcription regulation, cannot be excluded and requires further examination.

4.5 A novel role of HPr phosphorylation? pH-dependent control of carbon uptake

Even though the mechanism of HPr phosphorylation has already been extensively studied, only little is known about the physiological role of this conserved mechanism in *M. pneumoniae*. This work finally confirms that the phosphorylation state of HPr is not involved in transcription regulation, which is in good agreement with proteome analyses of *hprK* and *prpC* mutants (Schmidl *et al.*, 2010). However, this finding is not too surprising since no transcription regulator responsive to HPr phosphorylation such as CcpA is present in the genome of *M. pneumoniae.* In fact, CcpA orthologues are missing in all *Mollicutes* with the exception of *A. laidlawii*, which is more closely related to the *Firmicutes*. In contrast, HPr kinase is present in the genome of the majority of the *Mycoplasmas*, but absent in the *Phytoplasma* subgroup. Thus, just like RelA as discussed above, the phosphorylation state of HPr plays a role in the *Mollicutes* distinct from regulating transcription as in the *Firmicutes*. In this work, evidence is provided that HPr phosphorylation is involved in regulating the uptake of glucose and other PTS sugars in a pH-dependent manner: whereas the wild type stops to acidify the medium by metabolizing glucose at a certain pH, an *hprK* mutant is consuming all glucose available and acidifies the medium to a much lower pH. In contrast, a *prpC* mutant stops metabolizing glucose at a much higher pH compared to the wild type. The inability of HPr(Ser-P) to participate in phosphotransfer for glucose uptake due to conformational changes of the protein has been described in *B. subtilis* previously (Reizer *et al.*, 1989). Because of the high degree of structural homology, it is tempting to speculate that phosphorylation of Ser46 has the same structural consequences in HPr from *M. pneumoniae.* Hence, it seems that either phosphorylation or dephosphorylation of HPr at Ser46 is regulated in a pH dependent manner, thereby inhibiting glucose uptake to prevent over-acidification of the medium. This hypothesis is in good agreement with the finding that re-buffering of *M. pneumoniae* cultures to basic pH leads to complete consumption of glucose, whereas glucose uptake stops at a certain pH if no buffering occurs (Judith Wodke, CRG Barcelona, personal communication).

In other Gram-positive bacteria such as *B. subtilis*, different phosphorylated forms of HPr interact with proteins or are involved in phosphorylation events (other than PTS), both aiming to trigger enzymatic activities. However, in addition to HPr, no additional phosphorylation events related to HPrK were observed in *M. pneumoniae* in previous studies (Schmidl *et al.*, 2010). Moreover, no putative interaction partners despite PTS related enzyme II and HprK were identified in a global interactome study in *M. pneumoniae* (Kühner *et al*., 2009),

suggesting that pH dependent regulation of glucose uptake is the only function of HPr(Ser-P) in this organism.

4.6 Transcription regulation of the *rpsJ* operon by WhiA: a general case?

Among all strains investigated by microarray analysis in this work, only the *mpn241/whiA* exhibited a phenotype that met all criteria for direct transcription regulation described in chapter 4.2: in this strain, a constitutive up-regulation of the *mpn164*/*rpsJ* operon consisting of 25 genes was observed when compared to the wild type. Moreover, whereas transcription of this operon constantly decreased in the wild type along the growth curve as already described previously (Güell *et al.*, 2009), transcription intensity in the *mpn241/whiA* mutant stayed constantly high. Because of the predicted helix-turn-helix motif of Mpn241/WhiA and the *de facto* helix-turn-helix domain of its orthologue in *T. maritima* (Kaiser *et al.*, 2010) it is tempting to speculate that Mpn241/WhiA indeed is a DNA-binding transcription regulator, responsible for the down-regulation of the *rpsJ* operon in the stationary phase of growth.

Previous publications show that the *rpsJ* operon, also referred to as S10-*spc*-alpha cluster, is highly conserved in both Gram-positive and Gram-negative bacteria (Coenye and Vandamme, 2005). In *E. coli*, transcription of this operon is feedback regulated by ribosomal protein L4 that causes premature termination at a specific site of the leader sequence (Lindahl *et al.*, 1983). Moreover, several other operons in *E. coli* that encode ribosomal proteins are feedback regulated by transcriptional or translational control, an important system that permits stoichiometric synthesis of ribosomal proteins and helps to maintain balance of ribosomal protein and rRNA levels (Zengel and Lindahl, 1994). However, neither ribosomal protein L4 nor other proteins involved in these regulatory mechanisms are encoded in the S10-*spc*-alpha cluster of Gram-positive bacteria including *M. pneumoniae*, making an involvement of these proteins in the observed regulatory effect unlikely. In general, little is known about regulation of ribosomal protein synthesis in Gram-positive bacteria. To date, only very few mechanisms have been described, among them stringent control mediated by (p)ppGpp and the feedback autoregulation of ribosomal protein RpsD in *B. subtilis* (Grundy and Henkin, 1991; Eymann *et al.*, 2002). Because controlled expression of ribosomal components is required for growth or complex cellular processes such as sporulation in *B. subtilis*, it might be possible that WhiA proteins are regulators that control the expression of the S10-*spc*-alpha cluster in Gram-positive bacteria (Nanamiya *et al.*, 2010; Nanamiay and Kawamura, 2010).

Hence, if WhiA proteins are responsible for the regulation of the *rpsJ* operon in all Gram-positive bacteria by binding to an operator sequence, a common DNA motif should be found upstream of this operon in all these bacteria. Interestingly, comparisons of the transcriptional start sites of the *rpsJ* operon from *M. pneumoniae* and *B. subtilis* show that their promoters are located more then 120 bp upstream of the first start codon, leading to unusually long leader transcripts (Li *et al.*, 1997; Marc Güell, CRG Barcelona, personal communication). Thus, a common DNA motif putatively targeted by WhiA proteins might be located within this region.

A

Name	Start	p-value	Sites
bce	102	6.39e-23	CGATGAAGTG GAAGGTTGCTGACACACCCGGCCGCTTTGCCATGG CATGGTGTGG
bli	101	3.13e-22	CGATGAAGTG AGAGGTTGCTGACACACCCGGCCGCTTTGCCATGG CAAGGTGATT
bsu	101	3.13e-22	CGATGAAGTG AGAGGTTGCTGACACACCCGGCCGCTTTGCCATGG CAAGGTGTTC
lin	100	3.13e-22	TGATGAAGTG AAAGGTTGCTGACACACCCGGCCGCTTTGCCATGG CGGATGTTCA
bpu	100	3.13e-22	CGATGAAGTG AGAGGTTGCTGACACACCCGGCCGCTTTGCCATGG CAAGGTGATC
lmo	100	3.13e-22	TGATGAAGTG AAAGGTTGCTGACACACCCGGCCGCTTTGCCATGG CGGATGTTCA
bcl	98	1.71e-21	CGATGATGCG GAAGGTTGCTGACACACCCGGCCCCTTTGCCATGG GAGGGTGAGG
lsa	201	4.45e-17	GCTAAGATGT GAGAGGTTGCGACACACCCGGCCGCTTTGCCATGG GCGGGCGTGC
mga	67	6.30e-13	GGCGTTTTGA AAAGATTGCTGATACACCAAAAACCGTTGTCAAGT ATCAGGTAAC
upa	112	1.88e-11	TTGTTATTGT TGAGATTGCTGATACGCCAAGAAACGTTGTCAATA AAACGTATCA
mpu	92	2.92e-11	CTTTGAGACA TAAGGTTGCTGATACACCAAAATAAGTTGTTCGTG GTTATCAGGT
lla	96	3.50e-11	GCGACGAAAC GAGAGGTTGCGACACACCCGAAGGTATTGCCATAC CTAACGTGTC
sco	102	1.94e-10	ATTCATGTCA GCGAGAGTGCGACACGCCCGACCGCGTGGGTCGGA GGGGGAGCGG
mpn	68	5.57e-10	TTAAGGCTTT GGTCGAAGTGGATACACCGAAAAGCGTAGTCATTT TGACCATCAA
mge	63	9.51e-10	TAACGCTTTA GTTTTTAGTTGATACACCAAAATCCGTAGTCAATT TATTAACTAA
mar	93	2.05e-09	ACATTGAAAC ATAAGGTTACGATACGCCGAGCTTAGTTGTTCACC TCTCGTATCG
mmy	60	5.42e-09	TATTTTGTGT GCTTTTTTACAACACACCCATAGGAGTTGTTGATT AAATTAGTAA

B

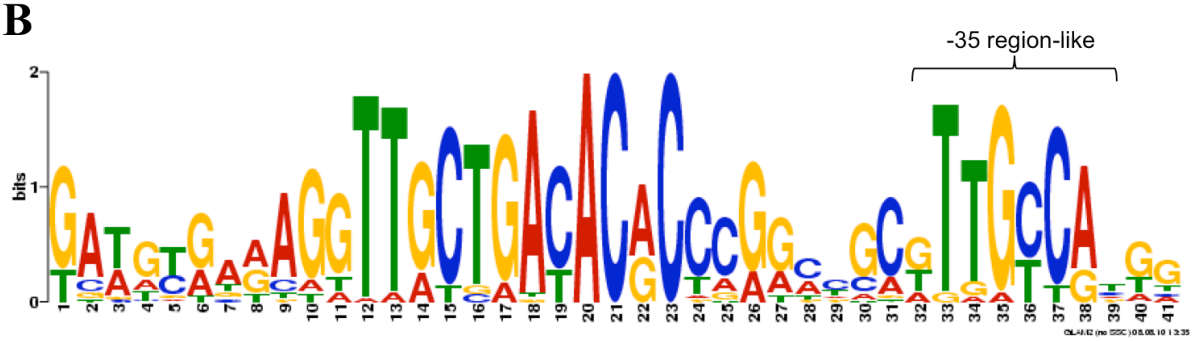

Fig. 4.4: Identification of a putative binding site for WhiA proteins upstream of *rpsJ*

A: Multiple alignments of the leader sequence of *rpsJ* from *B. cereus, B. licheniformis, B. subtilis, L. innocua, B. pumilus, L. monocytogenes, B. clausii, L. sakei, M. gallisepticum, U. parvum, M. pulmonis, L. lactis, S. coelicolor, M. pneumoniae, M. genitalium, M. arthritidis* and *M. mycoides* (top to bottom). A long, asymmetric DNA motif is conserved in the *Firmicutes* whereas only a gapped core motif is conserved in all sequences aligned. The motif is always located in a similar distance to the corresponding start codon of *rpsJ* (Start). B: MAST analysis of *rpsJ* leader sequences from 50 different bacteria possessing WhiA orthologues reveals the presence of a gapped, asymmetric core motif. The 3´ sequence of this motif strongly resembles sequences from -35 regions of bacterial promoters recognized by sigma70 factors, TTGACA.

To identify putative target sequences, multiple alignments of the up-stream regions of *rpsJ* from 8 *Firmicutes* and 8 *Mollicutes* in which WhiA orthologues occur, and that of *S. coelicolor* in which a WhiA phenotype has been described, were performed. As shown in Fig. 4.4, an asymmetric 47 bp long DNA sequence is conserved in a similar distance to the start codon of *rpsJ* in all *Firmicutes*, whereas this sequence is only weakly conserved in the *Mollicutes*. Hence, the low degree of conservation of this sequence could explain the relatively weak induction of transcription in strain GPM22 due to a lowered affinity of Mpn241/WhiA to this region. MAST analysis (http://meme.sdsc.edu) of sequences upstream of *rpsJ* from 50 different Gram-positive bacteria that harbour WhiA orthologues reveals the presence of an asymmetric, gapped core motif present in all sequences. Interestingly, the 3´ part of this motif strongly resembles DNA sequences of -35 regions of bacterial promoters recognized by sigma70 factors. Structural analysis of a WhiA orthologue from *T. maritima* demonstrated that WhiA proteins possesses a bipartite structure, consisting of two putative DNA binding domains: an N-terminal domain showing a LAGLIDADG homing endonuclease (LHE) scaffold and a helix-turn-helix domain that strongly resembles related regions of bacterial sigma70 factors responsible for the recognition of -35 regions of promoters (Kaiser *et al.,* 2009). Thus, it is tempting to speculate that this helix-turn-helix domain binds to the 3´ sequence of the discovered motif upstream of *rpsJ*. On the other hand, members of the LHE family bind to long, asymmetric DNA sequences that can be extremely degenerated by mutation (Gimble and Wang, 1996; Argast *et al.*, 1998). Thus, the LHE-like domain could be involved in binding the 5´ sequence of this motif. However, despite RecA proteins in *Mycobacteria* that have been shown to maintain their endoculease activity, no protein of this family has been described in bacteria so far and nothing is known about DNA sequences recognized by these proteins (Singh *et al.*, 2009). Therefore, further experiments are required to determine the prerequisites for DNA binding of WhiA proteins and the role of this DNA motif in the regulation of the S10-*spc*-alpha cluster in Gram-positive bacteria. Moreover, if WhiA proteins are acting as repressors, the mechanism by which the DNA binding activity of WhiA proteins is regulated requires further inestigations. Only very few possible protein interaction partners of Mpn241/WhiA have been discovered in a recent interactome study that could be involved in this mechanism. Among them were components of sugar transporters, enzymes of central metabolic pathways, translation elongation factor Tuf and DnaB involved in replication initiation, making several different mechanisms of regulation thinkable (Kühner *et al.*, 2009).

The name WhiA is derived from the phenotype in *S. coelicolor* that forms white colonies if the orthologous gene is disrupted. Therefore, if further experiments indeed show that WhiA proteins are conserved regulators of the S10-*spc*-alpha cluster in Gram-positive bacteria, an alternative name is proposed for these proteins: transcriptional regulator of ribosomal proteins, TrrP.

5. Outlook

Two of the main questions addressed in this work were (I) what is the minimal requirement of regulation of gene expression in a minimal organism, and (II) how does *M. pneumoniae* accomplish the massive transcription regulations observed previously (Güell *et al.*, 2009) with such a little set of transcription regulators? This work shed some light at least on the first question by proofing the essentiality of some genes putatively involved in transcription regulation and by verifying the involvement of other genes in regulatory processes. However, several further questions result from this work. Because target genes of the putative, essential transcription regulators could not be identified in this work, their precise implication in transcription regulation is still not clear. Thus, alternative approaches such as ChIP-chip need to be developed for *M. pneumoniae* to identify targeted genes. Moreover, even though it could be shown that the mechanism leading to (p)ppGpp formation is conserved in *M. pneumoniae*, no phenotype could be observed, suggesting that *M. pneumoniae* lacks a stringent response known in other bacteria. RelA proteins are conserved in all facultative intracellular pathogens, but not in obligate intracellular pathogens. Furthermore, all *relA* mutants of pathogenic bacteria published to date are impaired in infection or host adaption. Hence, a proper infection model needs to be developed to proof an involvement of *M. pneumoniae* RelA in host adaptation.

A participation of HPr(Ser-P) in transcription regulation could be excluded by the results of this work. However, evidence for a pH dependent feedback mechanism of HPr(Ser46) phosphorylation was provided leading to a stop of glucose uptake. To verify this mechanism, native blots using cell extracts have to be performed to proof a pH dependent phosphorylation state of HPr in *M. pneumoniae in vivo*. Moreover, *in vitro* phosphorylation experiments could be performed to study if the activity of HPrK is directly triggered by the pH or by an alternative mechanism including additional factors, such as proteins.

Finally, experiments are required that proof that TrrP proteins (formerly WhiA) are conserved regulators of the S10-*spc*-alpha cluster in other Gram-positive bacteria. Therefore, *trrP* deletion mutants of other bacteria need to be constructed and the effect on transcription of the S10-*spc*-alpha cluster has to be studied. Such experiments are currently ongoing in the *Firmicute B. subtilis*. Moreover, experiments have to be performed to proof that TrrP binds to the conserved motif upstream of *rpsJ*. To achieve this, EMSA experiments using TrrP and DNA fragments containing *rpsJ* leader sequences from different bacteria could be performed. Mutational analysis of the conserved core motif and DNase foot-printing experiments would

then provide final evidence for a specific interaction of TrrP with this motif. However, all these proposed experiments only shed little light on transcription regulation in *M. pneumoniae*. To get a much deeper insight into regulation of gene expression, the role of small non-coding RNAs needs to be studied, as well as RNA turnover and RNA processing since nothing is known about these regulatory events in *M. pneumoniae*.

6. Summary

Mycoplasma pneumoniae has maintained only a small repertoire of genes putatively involved in transcription regulation. This reflects its adaption to a nutrient rich and rather constant habitat, the human lung epithelium. To date, the heat shock response regulator HrcA is the only characterized protein involved in transcription regulation in this organism. However, several transcription regulation events have been described, leading to the question which mechanisms or which regulators are responsible for these events. This work aimed to study the implication of the putative, so far uncharacterized regulators in transcription in *M. pneumoniae*. Screening of a mutant library showed that the majority of the genes encoding these regulators are essential for viability. Only mutants affected in genes *relA* and *whiA,* both widely distributed and highly conserved in bacteria, could be isolated from the library. In addition, interaction of protein Mpn266 with RpoA could be shown, providing evidence that this protein is an orthologue of transcription regulator Spx in the *Firmicutes*. However, this regulator is essential, too. Several experiments have been performed to characterize the role of RelA and the alarmone (p)ppGpp in *M. pneumoniae*. Whereas it could be shown that mechanisms leading to (p)ppGpp formation in this organism are conserved, no additional phenotype could be identified in a *relA* mutant despite an inability to synthesize these alarmones. An effect on transcription regulation could not be shown by microarray analysis. However, evidence was provided that (p)ppGpp formation is not involved in short-term regulation of transcription in *M. pneumoniae*. Experimental data indicate that the phosphorylation state of HPr is involved in regulating the uptake of glucose and other PTS sugars in a pH dependent manner, rather then in transcription regulation. Microarray analysis of a *whiA* mutant showed that a large operon consisting mainly of genes encoding ribosomal proteins is constitutively up-regulated in this strain. Both the regulator and the operon co-occur in Gram-positive bacteria, suggesting a similar function of this regulator in other bacteria. The finding of a conserved DNA sequence upstream of this operon supports this hypothesis. In summary, this work provides the first microarray analyses of *M. pneumoniae* mutants. Furthermore, the first *M. pneumoniae* mutant impaired in transcription regulation and a novel putative role of HPr phosphorylation were described. Thus, this work provides a basis for future experiments that could help to understand regulatory mechanisms in this minimal organism.

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Ohne Moos nix los? Es soll deswegen nicht unerwähnt bleiben dass die **GGNB** (nicht nur) meinen Forschungsaufenthalt in Barcelona finanziell unterstützt hat.

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„Doktor Hinnerk Eilers, das klänge doch sehr gut.“ war ein Satz den mein während meines Studiums verstorbener **Vater** in meiner Kindheit einige Male angebracht hatte. Sowohl er als auch meine **Mutter** haben mich bis heute immer in allem was ich gemacht habe uneingeschränkt unterstützt. Aus diesem Grunde ist ihnen beiden diese Arbeit gewidmet.

Kerstin. Am schwierigsten fällt es mir zu schreiben wie sehr ich Dir danke. Nicht, weil ich nicht weiß was ich schreiben soll, sondern weil kein Blatt Papier groß genug wäre um dies alles zu fassen. Ohne Dich gäbe es diese Arbeit nicht. Ich liebe Dich.

9. Appendix

9.1 Materials

9.1.1 Chemicals

Chemical	Source
2,2'-dipyridyl	Fluka, Buchs, Switzerland
3-(N-morpholino)propanesulfonic acid (MOPS)	Fluka, Buchs, Switzerland
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Roth, Karlsruhe
5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid (X-Gal)	PeqLab, Erlangen
8-Bromoadenosine 5′-triphosphate sodium salt	Sigma-Aldrich, München
Acrylamide	Roth, Karlsruhe
Agar	Roth, Karlsruhe
Agarose	PeqLab, Erlangen
Ammonium iron (III) citrate	Sigma-Aldrich, München
Ammonium Peroxydisulfate (APS)	Roth, Karlsruhe
Antibiotics	Applichem, Darmstadt
Blocking reagent	Roche Diagnostics, Mannheim
Bowine serum albumine	MBI Fermentas, St. Leon-Rot
Bromphenol blue	Roth, Karlsruhe
CDP*	Roche Diagnostics, Mannheim
Coomassie blue, G250	Roth, Karlsruhe
D-Glucose	Roth, Karlsruhe
Desthiobiotin	IBA, Göttingen
Diethylpyrocarbonate (DEPC)	Roth, Karlsruhe
DL-Dithiotreitol	Sigma-Aldrich, München
DL-serine hydroxamate	Sigma-Aldrich, München
dNTPs	Fermentas, Lithuania
Ethidium bromide	Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, München
Glycerol	Merck, Damstadt
Immidazole	Sigma-Aldrich, München

Chemical	Source
Immidazole	Sigma-Aldrich, München
Isopropyl-ß-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich, München
Ni^{2+}-nitrilotriacetic acid superflow	Qiagen, Hilden
Nutrient Broth	Roth, Karlsruhe
Paraformaldehyde	Roth, Karlsruhe
Phenol red	Roth, Karlsruhe
Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES)	Roth, Karlsruhe
Potassium acetate	Merck, Damstadt
Potassium glutamate	Roth, Karlsruhe
PPLO broth	Becton, Dickinson & Company, France
Skim milk powder	Oxoid, Hampshire, UK
Sodium citrate	Roth, Karlsruhe
Sodium Dodecyl Sulfate	Roth, Karlsruhe
Sodium hydroxide	Roth, Karlsruhe
Sodium succinate	Fluka, Buchs, Switzerland
ß-Mercaptoethanol	Roth, Karlsruhe
Starch	Sigma-Aldrich, München
Strep-Tactin Sepharose	IBA, Göttingen
Sucrose	Sigma-Aldrich, München
Tetramethylethylenediamine (TEMED)	Roth, Karlsruhe
Tris(hydroxymethyl)aminomethane (Tris)	Roth, Karlsruhe
Tryptone	Oxoid, Hampshire, UK
Tween 20	Sigma-Aldrich, München
Xylencyanol	Merck, Damstadt
Yeast extract	Oxoid, Hampshire, UK

Tab. 9.1: Chemicals used in this study

Other chemicals were purchased from Fluka, Merck, Roth, Oxoid, Serva or Sigma-Aldrich. Compounds for the preparation of *M. pneumoniae* defined media (chapter 2.1.2) were provided by the CRG, Barcelona.

9.1.2 Auxiliary material and consumables

Material	Source
Cell culture flasks (75 cm^2, 150 cm^2, 300 cm^2)	Renner GmbH, Dannstadt
Cell culture plates (6 wells, 24 wells)	TPP AG, Switzerland
Cell scraper (240 mm)	Renner GmbH, Dannstadt
Centrifuge cups	Beckmann, Munich
Cuvettes (microlitre, plastic)	Greiner, Nürtingen
Dialysis tube	Roth, Karlsruhe
Electroporation cuvettes	Bio-Rad, Munich
Falcon tubes (15 ml, 50 ml)	Sarstedt, Nümbrecht
Gene amp reaction tubes	Applied Biosystems, USA
Glas pipettes	Ochs, Bovenden
Microlitre pipettes (2 µl, 200 µl, 1000 µl)	Gilson, Düsseldorf
Microtiter plates	TPP AG, Switzerland
Syringes, non-returnable	Becton Dickinson, Heidelberg
Nylon membrane, positively charged	Roche Diagnostics, Mannheim
Petri dishes	Greiner, Nürtingen
Pipette tips	Greiner, Nürtingen
	Eppendorf, Hamburg
	Sarstedt, Nümbrecht
Pipettes (2 µl, 20 µl, 200 µl, 1000 µl)	Eppendorf, Hamburg
Poly-Prep Chromatography columns	Bio-Rad, Munich
Polyvinylidendifluoride membrane (PVDF)	Bio-Rad, Munich
Serological pipettes	TPP AG, Switzerland

Tab. 9.2: Auxiliary material and consumables used in this study

9.1.3 Instrumentation

Device	Source
Biofuge fresco	Heraeus Christ, Osterode
Blotting device VacuGeneTMXI	Amersham, Feiburg
Chemiluminescence system ChemiSmart	PeqLab, Erlangen
CO_2 incubator C42	Labotect, Göttingen
Frensh pressure cell press	SLM Aminco, Lorch
Gel electrophoresis apparatus	PeqLab, Erlangen
Heatblock	Waasetec, Göttingen
HERA Safe Work Bench HS12	Heraeus Christ, Osterode
Hoefer TE 70 Semi Dry Blotting Device	Amersham, Feiburg
Horizontal shaker VXR basic	IKA, Staufen
Hydro tech vacuum pump	Bio-Rad, Munich
Ice maschine	Ziegra, Isernhagen
Image eraser	Molecular Dynamics, USA
Magnetic Stirer Reo basci C	IKA, Staufen
Mini-Protean III System	Bio-Rad, Munich
ND-1000 Spectrophotometer	Nanodrop Technologies, USA
OneShot Cell Disruptor	Constant Systems, UK
Orbital shaker G10	New Brunswick (Eppendorf), USA
OV2 Incubation oven	Biometra, Göttingen
pH meter	Knick Calimatic, Berlin
Refrigerated centrifuge	Kendro, Hanau
Special accuracy weighing machine	Sartorius, Göttingen
Spectral photometer	Amersham, Feiburg
Speedvac Univapo 150H	Zirbus technology, Bad Grund
Standard power pack	Bio-Rad, Munich
Steam autoclave	Zirbus technology, Bad Grund
Thermocycler iCycler	Bio-Rad, Munich
Thermocycler LabCycler	Sensoquest, Göttingen
Thermocycler Tpersonal	Biometra, Göttingen
Ultra centrifuge, Sorvall Ultra Pro 80	Thermo scientific, USA

Device	Source
Ultrasonic device UP200S	Dr. Hielscher, Teltow
UV Transilluminator Gel Doc 2000	Bio-Rad, Munich
Vibrax VXR basic	IKA, Staufen
Vortex	Bender & Hobein, Bruchsal
Water desalination plant	Millepore, Schwalbach
Water-bath incubation system	GFL, Burgwedel

Tab. 9.3: Technical equipment and instrumentation used in this study

9.1.4 Commercial systems

System	Source
Cy5™ Mono-Reactive Dye module	Amersham Biosciences
DNeasy Tissue Kit (50)	Qiagen, Hilden
iScript™ One-Step-RT-PCR	Bio-Rad, Munich
Merckoquant peroxide test	Merck, Darmstadt
Nucleospin Plasmid	Machery-Nagel, Düren
Qiaquick PCR Purification Kit	Qiagen, Hilden
RNeasy® Midi Kit	Qiagen, Hilden
RNeasy® Plus Mini Kit	Qiagen, Hilden
S.N.A.P.™ Purification module	Invitrogen
SuperScript™ Indirect cDNA Labeling System	Invitrogen

Tab. 9.4: Commercialized test systems and kits used in this study

9.1.5 Software

Name	Source	Description
ChemiCapt Version 11.07	Bio-Rad	Chemiluminescence analysis
FreeMind	Open source	Mind mapping
Microsoft Office 2007	Microsoft	Data processing
Nanodrop software	Nanodrop Technologies	Photometric measurements
Quantity One 4.1.0	Bio-Rad	Imaging of luminescence
R	Open source	Data processing
SeqManTM II 5.07	DNASTAR	Analysis of DNA sequencing files
Vector NTI	Invitrogen	DNA/protein sequence analysis

Tab. 9.5: Hard drive-based computer software used in this study

9.1.6 Internet based software, websites and databases

Name	Source	Description
http://www.subtiwiki.uni-goettingen.de/	Flórez *et al.*, 2009	Data base of *B. subtilis*
http://www.ncbi.nlm.nih.gov/pubmed		Literature research, general data base
http://www.genome.jp/kegg/kegg2.html	Ogata *et al.*, 1999	Data base of genes and genomes
http://cbi.labri.fr/outils/molligen/	Barré *et al.*, 2004	Data base of *Mollicutes*
http://genolist.pasteur.fr/Colibri/	Médigue *et al.*, 1993	Data base of *E. coli*
http://genolist.pasteur.fr/SubtiList/	Moszer *et al.*, 2002	Data base of *B. subtilis*
http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi	JCVI	Data base of genes and genomes
http://bips.u-strasbg.fr/EMBOSS/	EMBOSS	Molecular biology tools
http://multalin.toulouse.inra.fr/multalin/multalin.html	Corpet, 1988	Multiple alignment tool
http://www.chemie.fu-berlin.de/chemistry/general/units.html	FU Berlin	Unit calculations
http://expasy.org/	#	Data base of proteins families
http://rna.lundberg.gu.se/cutter2/		DNA restriction analysis
http://transterm.cbcb.umd.edu/query.php	Kingsford *et al.*, 2007	Prediction of Rho-independent terminators
http://prodoric.tu-bs.de/	Grote *et al.*, 2009	Regulon prediction
http://pfam.sanger.ac.uk/	Finn *et al.*, 2009	Data base of proteins families

Name	Source	Description
http://www.cellbiol.com/scripts/complement/reverse_complement_sequence.html		DNA reverse-complement tool
http://www.basic.northwestern.edu/biotools/oligocalc.html	Kibbe, 2007	Calculator for oligonucliotide properties
http://meme.nbcr.net/meme4_4_0/intro.html	#	DNA motif prediction tools
http://molbiol-tools.ca/Promoters.htm	#	Tools to predict bacterial promoters
http://132.248.32.45:8080/cgi-bin/ribex.cgi	Abreu-Goodger and Merino, 2005	Riboswitch prediction tool
http://www.transcriptionfactor.org	Wilson *et al.*, 2008	Data base of transcription factors
http://string.embl.de/	Snel *et al.*, 2000	Protein association network tool

Tab. 9.6: Internet-based software and databases used in this study

Several related publications could be found on the respective websites.

9.2 Oligonucleotides

9.2.1 Available oligonucleotides used in this study

	Sequence[#]	Description*
SH29	ATGAGTGAGCTAACTCACAG	Sreening for *M. pneumoniae* mutants
SH30	CAATACGCAAACCGCCTC	Sreening for *M. pneumoniae* mutants
IW01	AAAGAATTCGATCAGCGGCTTC TGAAACGTG	Fwrd. Primer: amplification of P_{gltAB} from *B. subtilis*
IW02	AAAGGATCCTGAGCTTTTGGCAT TTGATTGTACGC	Rev. Primer: amplification of P_{gltAB} from *B. subtilis*
mls-fwd (kan)	CAGCGAACCATTTGAGGTGATA GGGATCCTTTAACTCTGGCAACC CTC	Construction *B. subtilis* mutants by LFH-PCR
mls-rev(kan)	CGATACAAATTCCTCGTAGGCG GGCCGACTGCGCAAAAGACATA AGCTCTCG	Construction *B. subtilis* mutants by LFH-PCR

Tab. 9.7: Oligonucleotides designed in previous works used in this study

restriction sites are underlined, introduced mutations are **bold**, promoters are *italic*, P indicates 5'-phosphorylation

* LFH-PCR: long flanking homology PCR

9.2.2 Oligonucleotides designed in this work

Name	Sequence[#]	Description*
HE167	AAAAGATCTATGATTGAAGCTAG TACAAAAAAGACAATGATTAAGG TCTTATCATTAC	Fwrd. primer: Amplification of BglII-P_{ackA}-STREP-MCS aus pGP1012
HE168	TTTTCTAGAATTCCCGGGATCCTT TTTCGAACTGCGGGTGGCTCCA**C** **CGCGG**CATT	Rev. primer: Amplifikation of BglII-P_{ackA}-STREP-MCS from pGP1012; mutagenesis PstI→ SacII
HE169	CACGCACATTAATAAGTTAGTGC	Fwrd. primer: Screening for Mpn317/*ftsZ* mutant
HE170	GCTTATTCTAAAGAAGAAAGACC	Rev. primer: Screening for Mpn317/*ftsZ* mutant

Name	Sequence[#]	Description*
HE171	AAAAAGCTTATGATTGAAGCTAG TACAAAAAAGAC	Fwrd. primer: Amplification of P_{ackA}-STREP from pGP1012
HE172	AAAGAATTCTTTTTCGAACTGCGG GTGGC	Rev. Primer: Amplification of P_{ackA}-STREP from pGP1012
HE173	AAATGATCAATGATTGAAGCTAG TACAAAAAAGAC	Fwrd. Primer: Amplification of P_{ackA}-STREP from pGP193
HE174	AAATCTAGAACTAGTGGATCCCC	Rev. Primer: Amplification of P_{ackA}-STREP from pGP193
HE175	AAAGAATTCATGAAGAAGATTCA AGTAGTCGTTAAAG	Fwrd. Primer: Amplification of Mpn053/*ptsH*
HE176	TTTGGATCCCTTAAATAACTTGGT GTTTTTCTAAAAC	Rev. Primer: Amplification of Mpn053/*ptsH*
HE179	CATTCCGGGATAATTTTCCCG	Fwrd. Primer: Screening for Mpn626 mutants
HE180	CAAGAAGAAGGATATCTTCATCT AG	Rev. Primer: Screening for Mpn626 mutants
HE181	GACAAGCATAATTATCCCGCTG	Fwrd. Primer: Screening for Mpn329 mutants
HE182	GCACTTAAGTGGTTGACAAACTG	Rev. Primer: Screening for Mpn329 mutants
HE183	AAAGGATCCGGCGTTAATAATGA TGATTGAAGC	Fwrd. Primer: Amplification of P_{ackA} from *M. pneumoniae*
HE184	TTTTGAATTCTTTTATCTAATAGG TAACAATAATAAAATATTAG	Rev. Primer: Amplification of P_{ackA} from *M. pneumoniae*
HE185	AAAGAATTCATGACAAAGCGTAA TAAAAAGAACAACAAGCTGTACA AG	Fwrd. Primer: Amplification of *rpoC* from *M. pneumoniae*
HE186	TTTCTAGATTAGTGATGGTGATGG TGATGATACTCCTCTTGAACCGTT TTGGCACCGAG	Rev. Primer: Amplification of *rpoC* from M. pneumoniae
HE187	CAATTGACGCAAGATCTTTTTAAC C	Rev. Primer: *rpoC* Sequencing

Name	Sequence[#]	Description*
HE188	GGTTACATCTTCCGGAACCTG	Fwrd. Primer: *rpoC* Sequencing
HE189	CCAGATCAGGTCGTCCTG	Rev. Primer: *rpoC* Sequencing
HE190	CCTTTTAGGTAAACGGGTGG	Fwrd. Primer: *rpoC* Sequencing
HE191	CTTGGAGATGACCTTCTTACC	Rev. Primer: *rpoC* Sequencing
HE192	GCATACGAAGCGGGCAAG	Fwrd. Primer: *rpoC* Sequencing
HE193	GTCATGTAACCAGACTTAGCC	Rev. Primer: *rpoC* Sequencing
HE194	GTGTCGAGTGAGATCCAGG	Fwrd. Primer: *rpoC* Sequencing
HE195	CTTCCTGCGCGTTTTGGG	Rev. Primer: *rpoC* Sequencing
HE196	CCCAGTTAACGATGCGTACC	Fwrd. Primer: *rpoC* Sequencing
HE203	TTTTGGATCCATGGCAAAGGA ATTAGTATTTGGC	Fwrd. Primer: Amplification of *groEL*
HE204	TTTTCTGCAGCTATTCTTGGA AGCTACCTTCC	Rev. Primer: Amplification of *groEL*
HE205	TTTTGGATCCATGAAAAATTT AACTACCCGACAAGCCCAAA TTC	Fwrd. Primer: Amplification of *HrcA*
HE206	TTTTCTGCAGTTAAGTGGAGC TAGTGGTTTTACCACCG	Rev. Primer: Amplification of *HrcA*
HE207	P-CTTACTGGAAGATACT AGTGTCTG**G**CAACAAATGGC CTTTATGAACCAAAACAACC	CCR mutagenesis primer for *hrcA* , 5´-phosphorylated
HE215	CCCATAGCTTTGGACACAC	pMT85/pGP194 sequencing; Fwrd. anneals upstream of SpeI
HE217	AAAGAATTCATGGTGCTGAA ATCGAAAGGATCC	Fwrd. Primer: Amplification of Mpn329
HE218	TTTTCTAGATTATTGATCATCT TCAAGTGTACCGC	Rev. Primer: Amplification of Mpn329
HE219	AAAGAATTCATGATTTCAGCG AAAGAGCAAGC	Fwrd. Primer: Amplification of Mpn239
HE220	TTTGCGGCCGCTTAAGCATTA ATAATTTTGATGCGGGG	Rev. Primer: Amplification of Mpn239

Name	Sequence[#]	Description*
HE221	AAAGGATCCATGATTTCAGCG AAAGAGCAAGC	Fwrd. Primer: Amplification of Mpn239
HE222	AAACTGCAGTTAAGCATTAAT AATTTTGATGCGGGG	Rev. Primer: Amplification of Mpn239
HE223	AAAGTCGACATGGTGCTGAA ATCGAAAGGATCCGTG	Fwrd. Primer: Amplification of Mpn329
HE224	AAACTGCAGTTATTGATCATC TTCAAGTGTACCGCTAGCGAC	Rev. Primer: Amplification of Mpn329
HE225	CTGATGAAAAACAACTAACG CAAATC	Fwrd. Primer: Promoter region of Mpn239
HE226	CCTAAGAGGGTATTGAGGAT AG	Rev. Primer: Promoter region of Mpn239
HE227	AGGTCATCCACTTAAACGAGT CC	Fwrd. Primer: Promoter region of Mpn329
HE228	CAAGTGGTGTTTGAGCTCAGC	Rev. Primer: Promoter region of Mpn329
HE229	P-CCAGGACAACAAATTGCCC AAGTG**G**GCTAAGCTCTCAACC	Mutagenesis primer for MMR of Mpn239; 5´-phosphorylated
HE230	CTAATAGCGCGTTTAGGTAGT C	Fwrd. Primer: Promoter region of *nox*
HE231	CCAAAAAGGAGATGTTGGTA TTGC	Rev. Primer: Promoter region of *nox*
HE232	CCAAAAATTCAATTAACCGGA AGTTTGC	Fwrd. Primer: Mpn517 probe
HE233	*CTAATACGACTCACTATAGGGA* *G*ACGTCAGTGATAAATGGCTG AAAATCAAAGCC	Rev. Primer: Mpn517 probe, contains T7-Promotor
HE234	AAAGGATCCATGAGTACCAA ACCGTTAATCTTATTG	Fwrd. Primer: Amplification of Mpn517
HE235	AAAGTCGACTTATATCAATTT TTGTACGTCAGTGATAAATGG	Rev. Primer: Amplification of Mpn517

Name	Sequence[#]	Description*
HE236	GCTGTTAGAAGAGCTACTGATGAG	Fwrd Primer: Primer: Promoter region of Mpn517
HE237	CAGCATTGAGTTGTTGTTCCAAAGC	Rev. Primer: Primer: Promoter region of Mpn517
HE238	GGCGTGCGTGTATACGCGTGCGC	Fwrd. Primer: Screening for Mpn352 (*sigA*) mutants
HE239	GGGTTTTTGCTGTAAGACTAAATTAGCAATTGTGC	Rev. Primer: Screening for Mpn352 (*sigA*) mutants
HE240	CCTATCACCTCAAATGGTTCGCTGCGTAAGTTTATAGCTGGATGAGTGCAGTTG	Rev. Primer: Upstream of *B. subtilis fur;* Kan-extension for LFH-PCR
HE241	GAGGCAGCACACCATCTTTCAGCAC	Fwrd. Primer: Upstream of *B. subtilis fur;* for LFH-PCR
HE242	GAAAAAGAAGCGGGGGCATCGATTG	Sequencing of upstream LFH fragment: *fur* from *B. subtilis*
HE243	CGAGCGCCTACGAGGAATTTGTATCGGGAAATTTAAGATTAAAGATCATAGATTGACG	Fwrd. Primer: downstream of *B. subtilis fur;* Kan-extension for LFH-PCR
HE244	CAGGTTCTTCCAAAATCCCTGGCGG	Rev. Primer: downstream of *B. subtilis fur;* for LFH-PCR
HE245	GGAGTCAGCTCTTTCTTAATGCCGGC	Sequencing of downstream LFH fragment: *fur* from *B. subtilis*
HE246	AAACCCGGGAAAGGAGGAAACAATCATGGTGCTGAAATCGAAAGGATCCGTGCTTGATT	Fwrd. Primer: Amplification of Mpn329 for expression in *B. subtilis*
HE247	TTTTCTAGATTATTGATCATCTTCAAGTGTACCGC	Rev. Primer: Amplification of Mpn329 for expression in *B. subtilis*
HE248	AAAGAATTCCCACCTTTGATCACGATAAAAGAGAAT	Fwrd. Primer: Amplification and cloning of P_{Mpn517}
HE249	AAAGGATCCAGTTAGCTAACAATAAGATTAACGGTTTG	Rev. Primer: Amplification and cloning of P_{Mpn517}

Name	Sequence[#]	Description*
HE250	P-GCTACTGATGAGTTTTATA AGGAAAA**TTG**ACAAGCAAAA ATCAATGAACTTATTAAGTTG	Mutagenesis of P_{Mpn517} (agc→ttg, optimized -35 region)
HE251	CTCCTTACGCATCTGTGCGG	Sequencing of pGlt-Kan derivates
HE252	GGCAGACATGGCCTGCCC	Sequencing of pGlt-Kan derivates
HE253	AAAGAATTCTTTGGCTTCCAA AACCAAACTACCC	Fwrd. Primer: Amplification of P_{nox}
HE254	AAAGGATCCGGTATTGCGATC ATAAGCGTTGAC	Rev. Primer: Amplification of P_{nox}
HE255	P-GGTAAAGCAAAGCTACAA CCAAAT**T**TG**A**CAACCAAGATC TTGCTCCACTATTAAATAAAT	Mutagenesis of P_{nox} (gtggca→ttgaca), optimized -35 Region
HE256	AAACCCGGGAAAGGAGGAAA CAATCATGTCATCGCCAAAGA AAAATTTCAAAAAACCTC	Fwrd. Primer: Amplification of Mpn352/*sigA*
HE257	AAATCTAGACTAATCCTTCTT TTCATTTCCCCTC	Rev. Primer: Amplification of Mpn352/*sigA*
HE258	AAACCCGGGAAAGGAGGAAA CAATCATGGCTGATAAACAA ACCCACGAGACAGAATTAAC	Fwrd. Primer: Amplification of *B. subtilis sigA*
HE259	AAATCTAGATTATTCAAGGAA ATCTTTCAAACGTTTACTTCT GC	Rev. Fwrd. Primer: Amplification of *B. subtilis sigA*
HE260	GTCAGAGTTGCAATGATTCCT GACGGATTGG	Fwrd. Primer: Upstream of *B. subtilis sigA;* for LFH-PCR
HE261	CCTATCACCTCAAATGGTTCG CTGCCAGACTCTGTTAATTGC TCTTTTACTTGGTCG	Rev. Primer: Upstream of *B. subtilis sigA*; Kan-extension for LFH-PCR
HE262	GCTATGACTCTGATAAAGCCG GTTATG	Sequencing of upstream LFH fragment: *sigA* from *B. subtilis*

Name	Sequence[#]	Description*
HE263	CGAGCGCCTACGAGGAATTTG TATCGCGTATTCGACAAATCG AAGCCAAAGCGTTGC	Fwrd. Primer: downstream of *B. subtilis sigA;* Kan-extension for LFH-PCR
HE264	GCACCGCATAACAAGGAAGG TAGGCGTGG	Rev. Primer: Upstream of *B. subtilis sigA;* Kan-extension for LFH-PCR
HE265	GCAGACAAAAATGGGCCGTC TGTTATTTCTCCG	Sequencing of downstream LFH fragment: *sigA* from *B. subtilis*
HE266	TTTTCTAGAGCAAAGCGCACA CTGCTATTATTG	Fwrd. Primer: Upstream of Mpn053/*ptsH*
HE267	AAA*GGATCC*TTATTTTTCGAA CTGCGGGTGGCTCCAAATAAC TTGGTGTTTTTCTAAAACTGC	Rev. Primer: Mpn053/*ptsH* with STREP-tag.
HE268	CAGTGAATTGTTTGAGTTTGT ACTGGAACAAATGG	Fwrd. Primer: Mpn395 probe
HE269	CTAATACGACTCACTATAGGG AGACAATAACCCACAGTTTGA CTACCTAAACGC	Rev. Primer: Mpn395 probe, contains T7 Promoter
HE270	CGCTTTTTACCTCATGAATCTT TCCCGAATCATG	Fwrd. Primer: Mpn393/*pdhA* probe
HE271	CTAATACGACTCACTATAGGG AGACTCGCAATTAAGTCATTA CCGTCAACGCG	Rev. Primer: Mpn393/*pdhA* probe contains T7 Promoter
HE272	GCTTGGGGTTGAAAATCAACG GTAATTG	Fwrd. Primer: Screening for Mpn266 mutants
HE273	CCTTTTCATTAAACGATGTGT AAAAACCTAG	Rev. Primer: Screening for Mpn266 mutants
HE274	AAATCTAGAGATGGTTACACT ATACACATCACCAAGC	Fwrd. Primer: *spx* from *B. subtilis* for B2H
HE275	AAAGGTACCCGGTTTGCCAAA CGCTGTGCTTCTC	Rev. Primer: spx from *B. subtilis* for B2H

Name	Sequence[#]	Description*
HE276	AAATCTAGAGATGATCGAGATTGAAAAACCAAAAATCGAAAC	Fwrd. Primer: *rpoA* from *B. subtilis* for B2H
HE277	AAAGGTACCCGATCGTCTTTGCGAAGTCCGAGTC	Rev. Primer: *rpoA* from *B. subtilis* for B2H
HE278	AAATCTAGAGATGCTTAAGAAAAAAGTTAATAATGATGCTGG	Fwrd. Primer: Mpn266/*spx* for B2H
HE279	AAAGGTACCCGCTTCTTTACTGTACGCACTTTAGG	Rev. Primer: Mpn266/*spx* for B2H
HE280	AAATCTAGAGATGGAAAAGTTTTTAAAGTACGAAATAAAGG	Fwrd. Primer: Mpn191/*rpoA* for B2H
HE281	AAAGGTACCCGAGATCTTAATTTGAGTCCTAATTCGTG	Rev. Primer: Mpn191/*rpoA* for B2H
HE282	P-CGAACCACCACTAGAACGTTG**G**CCATTGTTAACGGTAACAGCTG	CCR primer: mutation of Mpn191/*rpoA* (TGA→TGG);
HE283	AAAGGATCCAAAGGAGGAAACAATCATGCTTAAGAAAAAAGTTAATAATGATGCTGG	Fwrd. Primer: Amplification of Mpn266/*spx*
HE284	AAAAAGCTTCTTCTTTACTGTACGCACTTTAGGTTCC	Rev. Primer: Amplification of Mpn266/*spx*
HE285	GATCTAACTCCTTGTTAGCGTC	Fwrd. Primer: Screening for Mpn397*relA* mutants
HE286	GGCTATTGAAGAAACACTTAGTG	Rev. Primer: Screening for Mpn397*relA* mutants
HE287	AAAGCGGCCGCATGAAAAATTTAACTACCCGACAAGCCC	Fwrd. Primer: Amplification of Mpn124/*hrcA* with C-terminal STREP-tag

Name	Sequence[#]	Description*
HE289	AAAGCGGCCGCATGATTTCAGCGAAAGAGCAAGCTAAAAAAGG	Fwrd. Primer: Amplification of Mpn239/*gntR* with C-terminal STREP-tag
HE291	AAAGCGGCCGCATGGTGCTGAAATCGAAAGGATCCG	Fwrd. Primer: Amplification of Mpn329/*perR* with C-terminal STREP-tag
HE293	AAAGCGGCCGCATGTCCTTTAGTGTTCAGATTAAGCACGAG	Fwrd. Primer: Amplification of Mpn241/*whiA* with C-terminal STREP-tag
HE295	AAAGGATCCGTGTACCAAGAAGAAAGATTAGTAGCG	Fwrd. Primer: Amplification of *glcR* from *B. subtilis*
HE296	TTTAAGCTTTCAGTCCTTTCCTTCATCCTGCTCTG	Rev. Primer: Amplification of *glcR* from *B. subtilis*
HE297	AAAGGATCCAAAGGAGGAAACAATCGTGTACCAAGAAGAAAGATTAGTAGCG	Fwrd. Primer: Amplification of *glcR* from *B. subtilis*
HE298	TTTAAGCTTGTCCTTTCCTTCATCCTGCTCTGTC	Rev. Primer: Amplification of *glcR* from *B. subtilis*
HE299	TTTGAGCTCCTATTATTTTCGAACTGCGGGTGGCTCCAAGTGGAGCTAGTGGTTTTACCACCGTG	Rev. Primer: Amplification of Mpn124/*hrcA* with C-terminal STREP-Tag
HE300	TTTGAATTCCTATTATTTTCGAACTGCGGGTGGCTCCAAGCATTAATAATTTTGATGCGGGGGTTTA	Rev. Primer: Amplification of Mpn239/*gntR* with C-terminal STREP-Tag
HE301	TTTGAATTCCTATTATTTTCGAACTGCGGGTGGCTCCATTGATCATCTTCAAGTGTACCGCTAGC	Rev. Primer: Amplification of Mpn329/*perR* with C-terminal STREP-Tag

Name	Sequence[#]	Description*
HE302	TTTGAGCTCCTATTATTTTTCG AACTGCGGGTGGCTCCAATTT TGGTTTAGTTTTTTTAACTTAG CGTTGAG	Rev. Primer: Amplification of Mpn241/*whiA* with C-terminal STREP-Tag
HE303	GTAAACGGTTACATAAACAA GGAGGAGCTG	Frwd. Primer: qPCR for the promoter region of the *rbsR*-Operon from *B. subtilis* (*cre site*)
HE304	TCGCCGCTATGACACGCGTTC G	Rev. Primer: qPCR for the promoter region of the *rbsR*-Operon from *B. subtilis* (*cre site*)
HE305	GCAGTGAGAAAACGCTTATA AAACGAATGGAAG	Frwd. Primer: qPCR for the promoter region of the *purA*-Operon from *B. subtilis* (*cre site*)
HE306	GCACCTCCGTTAACCTTTCAA AACGATTC	Rev. Primer: qPCR for the promoter region of the *purA*-Operon from *B. subtilis* (*cre site*)
HE307	AAAAGATCTAAGCTTGCATGC CTGCAGGTCGAC	Rev. Primer: Amplification of $P_{[degQ36]}$-SD-STREP-MCS from pGP380 (with M13_puc_Fwrd)
HE308	AAAAGATCTTTATCATTTTTC GAACTGCGGGTGCTC	Rev. Primer zur Amplifikation $P_{[degQ36]}$–MCS-STREP-STOPSTOP from pGP382 (with M13_puc_Fwrd)
HE309	CTCAATTGGGAACAGTAAATG ATTGGTGG	Frwd. Primer: qPCR for the promoter region of Mpn674/*ldh*
HE310	ACCACGGCTCATGGCAGCGTA G	Rev. Primer: qPCR for the promoter region of Mpn674/*ldh*
HE311	ACAGCTGTCGAAACAGCAGC CAC	Frwd. Primer: qPCR for the promoter region of Mpn533/*ackA*
HE312	CCAGCATTGACTACTAAAATT TTGTTGTCGTTC	Rev. Primer: qPCR for the promoter region of Mpn533/*ackA*

Name	Sequence#	Description*
HE313	CAGTTAACTTGATTAACCACC ACTTTTGGAAC	Frwd. Primer: qPCR for the promoter region of Mpn434/*dnaK* (incl. CIRCE)
HE314	ACCAAGGTCAATGCCGATAAT TAAGCCG	Rev. Primer: qPCR for the promoter region of Mpn434/*dnaK* (incl. CIRCE)
HE315	GGACTTTGTGGAATCGATAAT TTTGAACCG	Frwd. Primer: qPCR for the promoter region of Mpn332/*lon* (incl. CIRCE)
HE316	CAGCTGGCATAGTTATTAAGA TAAGAGGTTG	Rev. Primer: qPCR for the promoter region of Mpn332/*lon* (incl. CIRCE)
HE317	AAAGAATTCGCAAAACTTATT AGTTAATCAACAG	Fwrd. Primer: Amplifcation of 16S rRNA promoters from *M. pneumoniae*
HE318	AAAGGATCCGACAGATTGCA TCCAGTTTTG	Rev. Primer: Amplifcation of 16S rRNA promoters from *M. pneumoniae*
HE319	P-CTGTATAATCTTCAG**A**CT GTTGACAACTCTGTC	CCR primer: mutagenesis of the 16S rRNA promoter from *M. pneumoniae* (+1G→+1A)
HE320	CGACCGACTCGACAACATCTC CTC	Fwrd. Primer: Mpn397/*relA* probe
HE321	CTAATACGACTCACTATAGGG AGAGACATAACCTCAACCCAT CGAGTTTG	Rev. Primer: Mpn397/*relA* probe, contains T7-Promotor
HE322	ATATATCATATGATTTCAGCG AAAGAGCAAGC	Amplification of Mpn239 (with HE300)
HE323	ATATATCATATGGTGCTGAAA TCGAAAGGATC	Amplification ofMpn329 (with HE301)
HE324	GGGTAACAATACCCTAAACG GCATAG	Fwrd. Primer: Screening for Mpn241 mutants
HE325	CAATTCCGCGGTCTTTGGTTT TGCG	Rev. Primer: Screening for Mpn241 mutants
HE326	CAGCTCAACGCCGAGCTAGA AC	Fwrd. Primer: Screening for Mpn244 mutants
HE327	CCCAAGATTATCAACTTAAGT GCCAAC	Rev. Primer: Screening for Mpn244 mutants

Name	Sequence#	Description*
HE328	CATTTGCGCAGCTGTTTCTCC ACG	Fwrd. Primer: Screening for Mpn273 mutants
HE329	CCAGTTATGAACCGTCCTACT CCC	Rev. Primer: Screening for Mpn273 mutants
HE330	TTTGAGAATTCAGCTCACACC CCG	Fwrd. Primer: Promoter region of *rpsJ* from *B. subtilis*
HE331	AAAGGATCCCGAATTTTTTGT TTTGCCATTATTTTCCC	Rev. Primer: Promoter region of *rpsJ* from *B. subtilis*

Tab. 9.8: Oligonucleotides designed and used in this study

restriction sites are underlined, introduced mutations are **bold**, promoters are *italic*, P indicates 5'-phosphorylation

* LFH-PCR: long flanking homology PCR

9.3 Plasmids

9.3.1 Available plasmids used in this study

Name	Resistance	Description*	Publication
p25-N	Kan^R	N-terminal fusions T25 domain, BACTH	EUROMEDEX#
pAC5	Amp^R/ Cm^R	Probing of translational *lacZ*-fusions in *B. subtilis*	Martin-Verstraete *et al.*, 1992
pAC6	Amp^R/ Cm^R	Probing of transcriptional *lacZ*-fusions in *B. subtilis*	Stülke *et al.*, 1997
pAC7	Amp^R/ Kan^R	Probing of translational *lacZ*-fusions in *B. subtilis*	Weinrauch *et al.*, 1991
pBGM31	Amp^R/Cat^R	Mutation of *B. subtilis*	Görke *et al.*, 2004
pBGM67	Amp^R/ Cm^R	Construction of a *B. subtilis yvcL* mutant	Görke *et al.*, 2005
pBlueskript	Amp^R	Cloning vector	STRATAGENE#
pBQ200	Amp^R/ Erm^R	Overexpression of proteins in *B. subtilis* under control of a strong *degQ36* promoter	Martin-Verstraete *et al.*, 1994

Name	Resistance	Description*	Publication
pClpB-TAP	Kan^R/ Gen^R	Expression of TAP-tagged proteins in *M. pneumoniae*	Kühner *et al.*, 2009
pET3c	Amp^R	Inducible over-expression vector for *E. coli*	NOVAGEN#
pGlt-Kan	Amp^R/ Kan^R	Shuttle vector that integrates in *gltA* of *B. subtilis*	Middleton and Hofmeister, 2004
pGP172	Amp^R	Inducible over-expression of proteins in *E. coli*; allows fusion to a Strep-tag at the N-terminus of the protein	Merzbacher *et al.*, 2004
pGP380	Amp^R/ Erm^R	Expression of proteins in *B. subtilis* allows fusion to a Strep-tag at the N-terminus of the protein	Herzberg *et al.*, 2007
pGP382	Amp^R/ Erm^R	Expression of proteins in *B. subtilis* allows fusion to a Strep-tag at the C-terminus of the protein	Herzberg *et al.*, 2007
pKT25	Kan^R	C-terminal fusions to the T25 domain, BACTH	EUROMEDEX#
pKT25-zip	Kan^R	Positive control for the BACTH	EUROMEDEX#
pMT85	Kan^R/ Gen^R	Transposon for the manipulation of *M. pneumoniae*	Zimmerman and Herrmann, 2005
pUT18	Amp^R	N-terminal fusions to the T18 domain, BACTH	EUROMEDEX#
pUT18C	Amp^R	C-terminal fusions to the T18 domain, BACTH	EUROMEDEX#
pUT18C-zip	Amp^R	Positive control for the BACTH	EUROMEDEX#
pWH844	Amp^R	Inducible over-expression of proteins in *E. coli*; allows fusion to a N-terminal His-tag	Schirmer *et al.*, 1997

Tab. 9.9: Plasmids constructed in previous studies used in this work

* Amp^R: ampicilin; Kan^R: kanamycin; Erm^R: erythromycin; Cm^R: chloramphenicol; Gen^R: gentamycin

Information about the plasmids related publications can be found on the website of the respective suppliers.

9.3.2 Plasmids constructed in this work

Name	Backbone	Description
pGP193	pBluSkP	P_{ackA}-His_6 from pGP1012,
	HindIII/EcoRI	HE171/HE172
pGP194	pMT85	P_{ackA}-$His_6$from pGP1012 with MCS from pGP193,
	SpeI/XbaI	HE173/174
pGP195	pMT85	P_{PackA}(-227 to -1) from *M. pneumoniae*,
	BamHI/EcoRI	HE183/HE184b
pGP196	pGP195	*rpoC*-His_6 from *M. pneumoniae*,
	EcoRI/XbaI	HE185/HE186
pGP197	pBluSkP	*rpoC*-His6 from *M. pneumoniae*,
	EcoRI/XbaI	HE185/HE186
pGP198	pWH844	*groEL* from *M. pneumoniae*,
	BamHI/PstI	HE203/HE204
pGP199	pBluSkP	*hrcA* from *M. pneumoniae*,
	BamHI/PstI	HE205/HE206
pGP199	pBluSkP	*hrcA* from *M. pneumoniae*,
	BamHI/PstI	HE205/HE206
pGP272	pWH844	Mutated *hrcA* from *M. pneumoniae*,
	BamHI/PstI	HE205/HE206/HE207]
pGP273	pGP194	*ptsH* from *M. pneumoniae,*
	EcoRI/BamHI	HE175/HE176
pGP274	pBluSkP	Mpn239,
	BamHI/PstI	HE221/HE222
pGP275	pWH844	Mutated Mpn239,
	BamHI/PstI	HE221/HE222/HE229
pGP276	pWH844	Mpn329,
	BamHI/PstI	HE223/HE224
pGP277	pBQ200	Mpn329 with RBS from pGP380
	XmaI/XbaI	
pGP278	pGlt-Kan,	P_{DegU}-RBS-Mpn329 from pGP277,
	EcoRI/XbaI	
pGP279	pAC6	$P_{Mpn517(nat)}$, native promoter region from Mpn517,
	EcoRI/BamHI	HE248/HE249

Name	Backbone	Description
pGP280	pAC6	$P_{Mpn517(mut)}$, modified promoter region from Mpn517,
	EcoRI/BamHI	HE248/HE249/HE250
pGP281	pAC6	$P_{nox(nat)}$, native promoter region from Mpn394/*nox*,
	EcoRI/BamHI	HE253/HE254
pGP282	pAC6	$P_{nox(mut)}$, modified promoter region from Mpn394/*nox*,
	EcoRI/BamHI	HE253/HE254/HE255
pGP283	pBQ200	Mpn352/*sigA* with RBS from pGP380,
	XmaI/XbaI	HE256/HE257
pGP284	pBQ200	*sigA* from *B. subtilis* with RBS from pGP380,
	XmaI/XbaI	HE258/HE259
pGP285	pMT85	Mpn053/*ptsH* with ~1kb upstream and 3´-STREP,
	BamHI/XbaI	HE266/HE267
pGP286	pAC6	*ldh* promoter from *M. pneumoniae*,
	EcoRI/BamHI	SH46/SH47
pGP287	pUT18,	*spx* from *B. subtilis,*
	XbaI, KpnI	HE274/HE275
pGP288	pUT18C,	*spx* from *B. subtilis,*
	XbaI, KpnI	HE274/HE275
pGP289	pKNT25,	*spx* from *B. subtilis,*
	XbaI, KpnI	HE274/HE275
pGP290	pKT25,	*spx* from *B. subtilis,*
	XbaI, KpnI	HE274/HE275
pGP291	pUT18,	*rpoA* from *B. subtilis,*
	XbaI, KpnI	H276/HE277
pGP292	pUT18C,	*rpoA* from *B. subtilis,*
	XbaI, KpnI	H276/HE277
pGP293	pKNT25,	*rpoA* from *B. subtilis,*
	XbaI, KpnI	H276/HE277
pGP294	pKT25, XbaI,	*rpoA* from *B. subtilis,*
	KpnI	H276/HE277
pGP295	pUT18,	Mpn266 from *M. pneumoniae,*
	XbaI, KpnI	H278/HE279

Name	Backbone	Description
pGP296	pUT18C, XbaI, KpnI	Mpn266 from *M. pneumoniae,* H278/HE279
pGP297	pKNT25, XbaI, KpnI	Mpn266 from *M. pneumoniae,* H278/HE279
pGP298	pKT25, XbaI, KpnI	Mpn266 from *M. pneumoniae,* H278/HE279
pGP299	pUT18, XbaI, KpnI	*rpoA* from *M. pneumoniae*, native, HE280/HE281
pGP300	pUT18, XbaI, KpnI	*rpoA* from *M. pneumoniae*, mutated (tga→tgg), HE280/HE281/HE282
pGP1451	pUT18C, XbaI, KpnI	*rpoA* from *M. pneumoniae*, mutated (tga→tgg) , HE280/HE281/HE282
pGP1452	pKNT25, XbaI, KpnI	*rpoA* from *M. pneumoniae*, mutated (tga→tgg) , HE280/HE281/HE282
pGP1453	pKT25, , XbaI, KpnI	*rpoA* from *M. pneumoniae*, mutated (tga→tgg) , HE280/HE281/HE282
pGP1454	pGP382, BamHI, HindIII	Mpn266 from *M. pneumoniae*, with SD from pGP380; HE283/HE284
pGP1455	pClpB-TAP, SacI, NotI	Mpn124/*hrcA*-STREP from *M. pneumoniae*, HE287/HE299
pGP1456	pClpB-TAP, EcoRI, NotI	Mpn239-STREP from *M. pneumoniae*, HE289/HE300
pGP1457	pClpB-TAP, EcoRI, NotI	Mpn329-STREP from *M. pneumoniae*, HE291/HE301
pGP1458	pClpB-TAP, SacI, NotI	Mpn241-STREP from *M. pneumoniae*, HE292/HE302
pGP1459	pGP882, BamHI, SmaI	*aphA3* (EcoRI/SmaI) and $P_{[degQ36]}$-SD-STREP-MCS (EcoRI/BglII); shuttle vector analogous to pGP380; integrates in *lacA* of *B. subtilis*
pGP1460	pGP882 BamHI, SmaI	*aphA3* (EcoRI/SmaI) und $P_{[degQ36]}$ –MCS-STREP-STOPSTOP (EcoRI/BglII); shuttle vector analogous to pGP382; integrates in *lacA* of *B. subtilis*

Name	Backbone	Description
pGP1461	pAC6 EcoRI/BamHI	Promoter of 16S rRNA from *M. pneumoniae*; HE317/HE318
pGP1462	pAC6 EcoRI/BamHI	Mutated Promoter of 16S rRNA from *M. pneumoniae*; HE317/HE318/ HE319
pGP1463	pMT85 BcuI / EcoRI	16S rRNA promoter-*lacZ* fusion, HE317/SH45
pGP1464	pMT85 BcuI /EcoRI	mutated 16S rRNA promoter-*lacZ* fusion, HE317/SH45
pGP1465	pET3c NdeI/EcoRI	Mpn239 with C-terminal STREP-tag, HE322/HE300
pGP1466	pET3c NdeI/EcoRI	Mpn329 with C-terminal STREP-tag; HE323/HE301
pGP1467	pAC7 EcoRI/BamHI	*rpsJ* promoter fragment from *B. subtilis,* HE330/HE331
pGP1468	pBGM31 SacI/BamHI, ApaI/KpnI	Contains flanking regions from *B. subtilis yvcL* derived from pBGM67, either SacI/BamHI or ApaI/KpnI digested

Tab. 9.10: Plasmids constructed in this work

9.4 Proteins, antibodies and enzymes

Description*	Source/Supplier
Anti-Digoxigenin-AP, Fab Fragments	Roche Diagnostics, Mannheim
DNase I	Sigma, München
Lysozyme from chicken egg protein (178000 U/mg)	Merck, Darmstadt
Phusion DNA Polymerase	Finnzymes, Finnland
Polyclonal AB against *M. pneumoniae* GlpK-STREP, (Hames *et al.*, 2005)	Seqlab, Göttingen
Restriction enzymes	NEB Biolabs, Frankfurt am Main MBI Fermentas, St. Leon-Rot
RNaseA	Roche Diagnostics, Mannheim
Secondary AB Anti-rabbit, coupled to IgG-AP gekoppelt	Promega, Mannheim
T4-DNA-Ligase	Roche Diagnostics, Mannheim
Taq polymerase, recombinant; purified from *E. coli* stock 147; purified by Julia Busse	Nadicom, Karlsruhe

Tab. 9.11: Enzymes, antibodies and other proteins used in this work

AB: antibody, AK: alkaline phosphatase

9.5 Bacterial strains

9.5.1 Available strains used in this study

Strain	Genotype	Reference
BL21	F$^-$,*lon ompT rBmB hsdS gal* (cIts857 *ind*1 Sam7 *nin5* *lac*UV5-T7 gene1)	Sambrook *et al.*, 1989
BTH101	F$^-$, *cya-99, araD139, galE15, galK16, rpsL1 (Str^R), hsdR2, mcrA1, mcrB1*	EUROMEDEX
DH5α	*recA1 endA1 gyrA96 thi hsdR17*rK- mK+*relA1 supE44* '80#*lacZ*#*M15* #(*lacZYA-argF*)U169	Sambrook *et al.*, 1989
XL1-Blue	*endA1, gyrA96(nal^R), thi-1, recA1, lac, glnV44*, F´[*::Tn10, proAB$^+$, lacI^q,* Δ*(lacZ)M15*] *hsdR17 (r_K^- m_K^+)*	STRATAGENE

Tab. 9.12: *E. coli* strains used in this study

Strain	Genotype	Reference
168	*trpC2*	Laboratory collection
HB0509	HB1000 *perR::spx*	Bsat *et al.*, 1998
HB6543	HB1000 *fur::kan*	Bsat *et al.*, 1998
GP278	*trpC2 amyE::(xynP-lacZ cat) xylR::Erm^R*	Singh *et al.*, 2007
ORB6781	*trpC2, pheA, spx::Spc^R*	P. Zuber, unpublished

Tab. 9.13: *B. subtilis* strains used in this study

Strain	Genotype	Reference
M129	Wild type (ATCC 29342)	Laboratory collection
GPM51	*hprK::Tn*	Halbedel *et al.*, 2006
GPM52	*glpD::Tn*	Hames *et al.*, 2009
GPM68	*prpC::Tn*	Halbedel *et al.*, 2006
GPM70	*mpn474::Tn*	Hegermann *et al.*, 2008

Tab. 9.14: *M. pneumoniae* strains used in this study

9.5.2 Strains constructed in this work

Strain	Genotype	Construction
GP868	*perR: :spc*, *fur*:*mls*	chrom. DNA HB0509→GP879
GP869	*perR::spc*, *fur*:*mls, gltA::*P_{degQ}*-mpn329*	pGP278→GP868
GP870	*perR::spc*, *fur*:*mls, gltA::pGltA-Kan*	pGlt-Kan→GP868
GP871	*perR::spc, fur:mls, gltA::*P_{degQ}*-mpn329, amyE ::*$P_{Mpn517(nat)}$*-lacZ*	pGP279→GP869
GP872	*perR::spc, fur::mls, gltA::pGlt-Kan, amyE ::*$P_{Mpn517(nat)}$*-lacZ*	pGP279→GP870
GP873	*perR::spc, fur::mls, gltA::*P_{degQ}*-mpn329,amyE ::*$P_{nox(nat)}$*-lacZ*	pGP281→GP869
GP874	*perR::spc, fur:mls, gltA::pGlt-Kan, amyE ::*$P_{nox(nat)}$*-lacZ*	pGP281→GP870
GP875	*perR::spc, fur:mls, gltA::*P_{degQ}*-mpn329, amyE ::*$P_{Mpn517(mut)}$*-lacZ*	pGP280→GP869
GP876	*perR::spc, fur::mls, gltA::pGlt-Kan, amyE ::*$P_{Mpn517(mut)}$*-lacZ*	pGP280→GP870
GP877	*perR::spc, fur::mls, gltA::*P_{degQ}*-mpn329,amyE ::*$P_{nox(mut)}$*-lacZ*	pGP282→GP869
GP878	*perR::spc, fur:mls, gltA::pGlt-Kan, amyE ::*$P_{nox(mut)}$*-lacZ*	pGP282→GP870
GP879	*fur ::mls*	LFH→WT168
GP880	*amyE ::*$P_{517(nat)}$*-lacZ*	pGP279→WT168
GP881	*amyE ::*$P_{nox(nat)}$*-lacZ*	pGP281→WT168
GP882	*amyE ::*$P_{ldh(nat)}$*-lacZ*	pGP286→WT168
GP883	*spx ::spc*	chrom. DNA ORB6781→ WT168
GP230	*amyE ::*P_{rosJ}*-lacZ*	pGP1467→WT168
GP231	*amyE ::*P_{rosJ}*-lacZ, yvcL::cat*	pGP1468→GP230

Tab. 9.15: *B. subtilis* strains constructed in this study

Strain	Genotype	Construction
GPM13	Tn[P_{clpB}-*hrcA*-STREP]::??	pGP1455→Wild type
GPM14	Tn[P_{clpB}-*hrcA*-STREP]::??	pGP1455→Wild type
GPM15	Tn[P_{clpB}-Mpn239-STREP]::??	pGP1456→Wild type
GPM16	Tn[P_{clpB}-Mpn239-STREP]::??	pGP1456→Wild type
GPM17	Tn[P_{clpB}-Mpn329-STREP]::??	pGP1457→Wild type
GPM18	Tn[P_{clpB}-Mpn329-STREP]::??	pGP1457→Wild type
GPM19	Tn[P_{clpB}-Mpn241-STREP]::??	pGP1458→Wild type
GPM20	Tn[P_{clpB}-Mpn241-STREP]::??	pGP1458→Wild type
GPM21	Tn::Mpn397/*relA*	Isolated by Haystack mutagenesis
GPM22	Tn::Mpn241/*whiA*	Isolated by Haystack mutagenesis

Tab. 9.16: *M. pneumoniae* strains constructed or isolated in this study

9.6 Microarray data

The microarray data from all experiments are available upon request. If required, please contact:

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9.7 Abbreviations

Abbreviation	Full text
% (v/v)	% (volume/volume)
% (w/v)	% (weight/volume)
°C	Degrees Celsius
A	Ampere
ADP	Adenosine diphosphate

Abbreviation	Full text
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BACTH	Bacterial adenylate cyclase based two-hybrid system
bp	Base pairs
CAA	Casamino acids
cAMP	Cyclic adenosine monophosphate
CAP	Cyclase associated protein
CCR	Combined chain reaction
CDP*	2-Chlor-5-(4-Methoxyspiro{1,2-Dioxetan-3,2‘-(5‘-Chlor)Tricyclo[3.3.1.13,7]Decan}-4-yl)-1-Phenylphosphate, sodium salt
ChAP	Chromatin affinity purification
ChIP	Chromatin immuno precipitation
Da	Dalton
DBD	DNA binding domain
DEPC	Diethylpyrocarbonate
DHAP	Dihydroxyacetone phosphate
Dig	Digoxigenin
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EI	Enzyme I (of PTS)
EII	Enzyme II (of PTS)
et al.	et alia (and others)
FBP	Fructose-1,6-bisphosphate
Fig.	Figure
g	Gram
GDP	Guanosine diphosphate

Abbreviation	Full text
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
h	Hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HPLC	High-performance liquid chromatography
IMP (dehydrogenase)	Inosine monophosphate (dehydrogenase)
IPTG	Isopropyl β-D-1-thiogalactopyranoside
l	Litre
LB	Luria Bertani (medium)
LC-M/S	Liquid chromatographie- mass spectrometry
LFH-PCR	Long flanking homology PCR
M	molar (mol/l)
min	Minute
mM	milimolar
MMR	Multiple mutation reaction
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide (reduced)
OD	Optical density
ORF	Open reading frame
PAA	Poly acryl amide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvic acid
pH	Power of hydrogen
PNPase	Polynucleotide phosphorylase
PPLO	Pleuropneumonia-Like organisms
PTS	Phosphotransferase system
qPCR	Quantitative PCR
PTS	Phosphotransferase system

Abbreviation	Full text
qPCR	Quantitative PCR
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Rounds per minute
rRNA	Ribosomal RNA
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	Seconds
Ser	Serine
SHX	Serine hydroxamate
sRNA	Small (non-coding) RNA
Tab.	Table
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
tRNA	Transfer RNA
U	Units
UV	Ultra violett (light)
V	Volt
WT	Wild type
X-Gal	Bromo-chloro-indolyl-galactopyranoside

Tab. 9.17: List of abbreviations used in this publication, in alphabetical order.

Curriculum vitae

Biographical data

Name:	Hinnerk Eilers
Date and place of birth:	March 20th 1980
	Seesen am Harz, Niedersachsen, Germany
Citizenship:	German
Marital status:	Single
Parents:	Else Annedore Eilers, maiden name Finke
	Hermann Eilers
Siblings:	Jan Frerich Eilers

Education

1986 – 1990	Elementary school, "Grundschule Münchehof", Seesen
1990 – 1992	"Orientierungsstufe", Seesen
1994 – 2000	Final school exams, "Jacobson Gymnasium", Seesen

Alternative civilian service

2000 – 2001	"Naturschutzstation Dümmer See", Hüde, Germany

Studies

2001 – 2002	Academic studies of political science and history, University of Hannover	
2004	Intermediate diploma in biology, University of Göttingen	
	Subjects:	Microbiology (main)
		Botany (subsidiary)
		Physics (subsidiary)
2006 – 2007	Diploma student in the group of Prof. Dr. Jörg Stülke, University of Göttingen	
2007	Diploma in biology, University of Göttingen	
	Subjects:	Microbiology (main)
		Phytomedicine (subsidiary)
		Organic chemistry (subsidiary)
2007 –2010	Ph. D. student in the group of Prof. Dr. Jörg Stülke,	
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Publications

Halbedel, S., Eilers, H., Jonas, B., Busse, J., Hecker, M., Engelmann S. and Stülke J., 2007. Transcription *in Mycoplasma pneumoniae*: analysis of the promoters of the *ackA* and *ldh* genes. *J. Mol. Biol.* 371:596-607.

Stülke, J., Eilers, H. and Schmidl, S. R., 2009. *Mycoplasma* and *Spiroplasma*, p. 208–219. *In* M. Schaechter (ed.), Encyclopedia of microbiology. Elsevier,Oxford, United Kingdom.