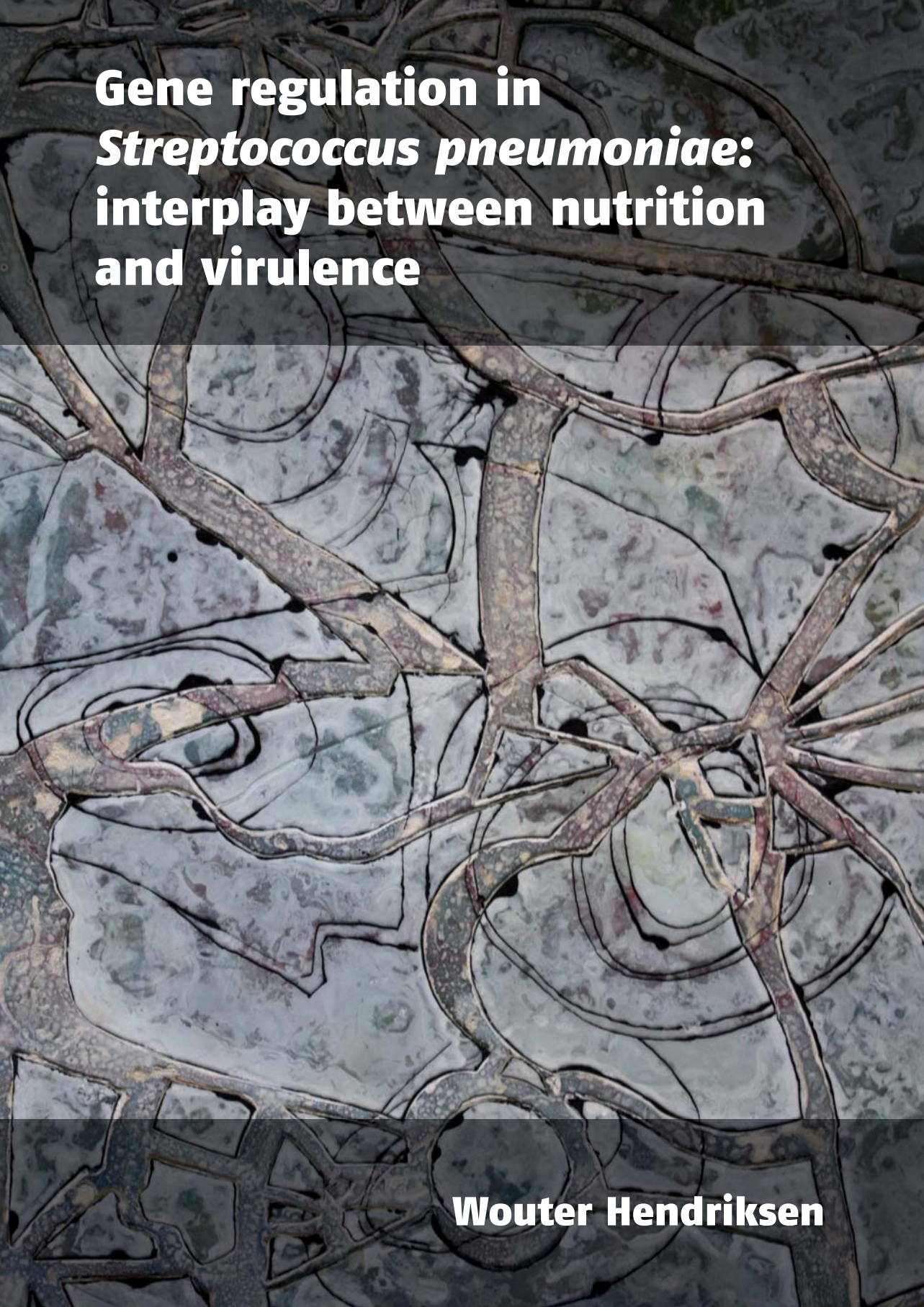
Gene regulation in *Streptococcus pneumoniae*: interplay between nutrition and virulence



Wouter Hendriksen

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Gene regulation in *Streptococcus pneumoniae*: interplay between nutrition and virulence

Genregulatie in Streptococcus pneumoniae: samenspel tussen voeding en virulentie

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CHAPTER 1

Introduction

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium, which belongs to the species of streptococci. Other pathogenic bacteria belonging to this class include *Streptococcus pyogenes, Streptococcus agalactiae*, *Streptococcus suis*, *Streptococcus uberis*, *Streptococcus bovis* and *Streptococcus mutans*. In addition, the pneumococcus is closely related to *Lactococcus lactis*, a non-pathogenic bacterium used for the production of dairy products. *S. pneumoniae*, formerly known as *Diplococcus pneumoniae*, was described for the first time in 1881, simultaneously by Louis Pasteur and George Sternberg. Since then, it has been under extensive study, and this research has significantly contributed to many fields in biology. The most well known example consists of the pioneering experiments of Griffith, who showed that non-virulent pneumococcal types could be transformed into virulent types using heat-killed virulent types (15), suggesting that transfer of hereditary features had occurred. This seminal observation was the basis that led to the discovery of DNA as the hereditary material (2).

Pneumococcal colonization and disease

Usually pneumococcus resides in the nasopharynx as a harmless commensal (Fig. 1). However, in children below the age of two years, immune-compromised persons, and elderly above the age of 65, the bacterium can cause serious infections such as otitis media, bacteremic pneumonia, meningitis, or bacteremia (Fig. 1). It is estimated that over 1 million children die from pneumococcal disease every year (6), most of them living in developing countries. In these countries medical aid is often only scarcely available, and the same is true for access to vaccines.

Invasive disease caused by pneumococci is always preceded by colonization in which the bacteria attach to the epithelial layer in the nasopharynx (Fig. 1). In susceptible individuals, colonization can lead to bacterial spread from the nasopharynx to the Eustachian tube, the lower respiratory tract, and the blood, causing serious infections (Fig. 1). A thorough understanding of the pneumococcal components required for pathogenicity and virulence is essential to combat disease. These components are often referred to as virulence factors, although many of these might contribute to virulence only indirectly, by affecting the overall fitness of the bacterium.

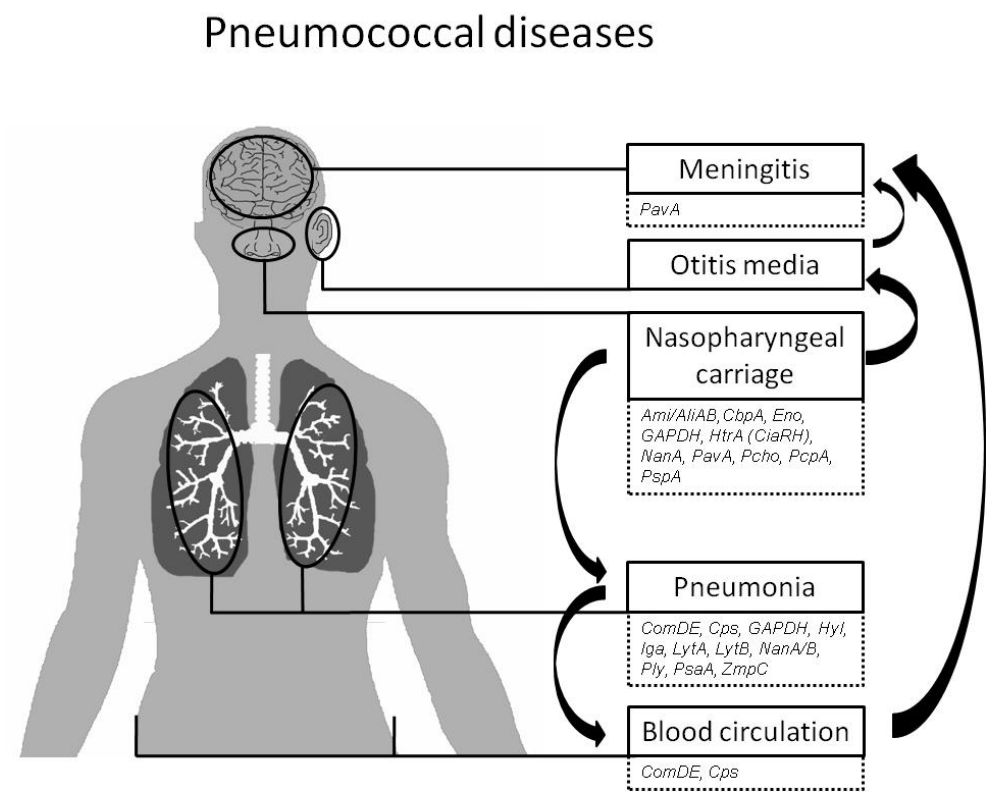

Figure 1. Infections caused by *S. pneumoniae*. Black arrows indicate the progression of infection. All pneumococcal diseases are preceded by nasopharyngeal carriage, after which the bacterium can spread to the middle ear causing otitis media or disseminate to the lungs causing pneumonia. During infection of the lungs, bacteria may penetrate into the blood circulation, from which they can spread through the whole body. Otitis media might also cause infection of the meninges. Some well-known examples of proteins, which have contributions to virulence at that particular site in the human or murine body, are indicated in dotted boxes. Ami-AliA/B, oligopeptide ABC transporter; CbpA, choline binding protein A; CiaRH, the Cia two-component system; ComDE, two-component system specific for sensing of competence stimulating peptide; Cps, polysaccharide capsule; Eno, α-enolase; GAPDH, glycerol-3-phophate dehydrogenase; HtrA, serine protease; Hyl, hyaluronate lyase; Iga, immunoglobulin A1 protease; LytA, autolysin A; LytB, autolysin B; NanA, neuraminidase A; NanB, neuraminidase B; PavA, pneumococcal adherence and virulence factor A; *P*Cho, phosphocholine; PcpA, choline binding protein PcpA; Ply, pneumolysin; PsaA, pneumococcal surface adhesin A [Mn^{2+} uptake ABC transporter]; PspA, pneumococcal surface protein A; ZmpC, metalloprotease.

Virulence factors

The most important virulence factor of pneumococcus is its capsule. This capsule is a shell of oligosaccharide molecules that forms a protective layer around the bacterium. The currently available pneumococcal vaccine is targeted at this sugar capsule. The proteins

responsible for the production and the structure of the capsule are encoded by the capsular polysaccharide synthesis (*cps*) locus. Up to now, over 93 different capsular types (serotypes) have been identified, which are distinct in biochemical structure. Recent research has shown that bacteria with distinct capsular structures also have large genetic differences in their *cps* locus (8). The role of the capsule is mainly a protective one, as it allows pneumococci to evade phagocytosis by immune cells. However, during colonization, the capsule might mask colonization factors, and regulation of the thickness of the capsule has been shown to occur during colonization (19).

Along with the oligosaccharide capsule, a cholesterol-dependent cytolysin called pneumolysin is regarded as the most prominent virulence factor (48). This protein has two important features; it can create pores in host cells and it can trigger the immune system (i.e., complement activation and pro-inflammatory responses) (11, 48).

The layer below the sugar capsule is the cell wall, consisting of peptidoglycan, teichoic and lipoteichoic acids, and phosphorylcholine. To this layer a variety of proteins with different functions are attached. Given that the outside of the cell wall, with all proteins attached, comes in close proximity with host cells during infection, many pneumococcal factors important for pathogenicity are found here.

In pneumococcus, outer membrane proteins can be classified into four groups on the basis of their attachment to the cell: proteins with an LPxTG motif, lipoproteins, choline binding proteins, and non-classical surface proteins (proteins lacking a classical leader sequence and membrane-anchoring motifs). Of these, a few examples with major roles in colonization or virulence are discussed below:

LPxTG proteins are covalently linked to the peptidoglycan after cleavage of their LPxTG motif by a sortase (47). Examples of LPxTG proteins are the neuraminidases NanA and NanB, which have an important role in cleaving sialic acid from host molecules such as mucin and glycoproteins (5, 23, 55). Hyaluronate lyase, which damages the extracellular matrix of human epithelial cells, also belongs to this class (33). Two other cell-sorted virulence factors containing the LPxTG motif, the metalloproteinases ZmpC and Iga1 protease cleave the extracellular matrix (10, 44) and the mucosal IgA1 antibody (50), respectively.

Phosphorylcholine, which is the anchor moiety for the group of choline binding proteins (CBPs), is anchored to the peptidoglycan and is also present in the plasma membrane (38). Major virulence factors belonging to the CBPs are the autolysins LytA, LytB, and LytC. These autolysins hydrolyze murein of the cell wall and are required for virulence (53).

Interestingly, autolysis induced by LytA is important for the release of the major virulence factor pneumolysin (36). The choline binding proteins CbpA, PspA, and PcpA have been linked to adhesion to host cells and to colonization in mice (35, 52, 54).

S. pneumoniae expresses over 42 lipoproteins, many of which play a role in virulence. For example, PsaA, part of a manganese uptake system, and the oligopeptide permease Ami/AliAB, are required for adhesion to host cells (1, 27). Interestingly, many of these lipoproteins seem to have a dual role, i.e., uptake of ions and/or nutrients and modulation of adhesion (directly or indirectly).

Examples of the non-classical surface-associated virulence factors with dual function are α-enolase and glycerol-3-phosphate dehydrogenase, both involved in carbohydrate metabolism (4, 18). In addition, PavA, pneumococcal adherence and virulence factor A, is a fibronectin adhesin, which is an important factor involved in adherence, invasion and modulation of meningal inflammation (21, 51). Together, these proteins form the most well known virulence factors that pneumococcus has, and are important for adhesion to host cells as well as bacterial survival within that host.

Expression of virulence factors

The expression kinetics of virulence factors during pathogenesis is largely unknown. Most likely, gene regulatory pathways and signal transduction play an important role in controlling the expression of these surface-exposed proteins. Consequently, expression studies to map gene expression pathways are of high importance, and are expected to contribute to the unraveling of the role of these proteins to the pathogenesis of pneumococcal colonization and infection.

Gene regulation

During their life cycle, bacteria often encounter changes in their environment. For pathogens in particular, this can be due to their migration to different sites in the human body. Usually, pneumococci reside in the nasopharynx where they cause no harm, but in the lungs they will cause serious infections. Clearly, adaptation to these changes is of utmost importance for bacterial survival. This adaptation is achieved by sensing the intracellular and/or extracellular environment and adjusting the transcription of genes to become most fit for the new or upcoming situation. This transcriptional adjustment will lead to production of new proteins, which will allow the pathogen to survive in its new environment. Transcriptional regulatory proteins of diverse functions orchestrate changes in gene

expression by activation or repression. Transcriptional regulators usually "sense" the intracellular concentration of particular ions or other molecules. When concentrations of these molecules reach a particular level, the regulatory protein will be activated or deactivated by, for instance, a conformational change induced as a result of binding of the stimulator molecule to the regulator. In addition to these transcriptional regulators, two-component signal transduction systems exist. These systems react on external signals, i.e., molecules or stimuli outside the cell and their regulation cascade is based on phosphorelay. Together, two component signal transduction systems and transcriptional regulators regulate global gene expression.

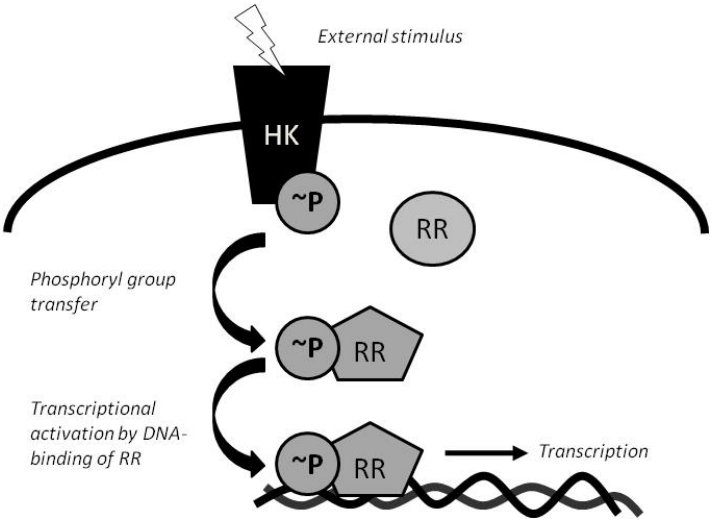

Figure 2. Schematic depiction of a two-component system. As a reaction on sensing its external stimulus, the histidine kinase (HK) is autophosphorylated. This phosphoryl group can then be transferred to the cognate cytoplasmatic response regulator (RR), which will undergo a conformational change. In this state, the phosphorylated RR can bind to DNA and control the expression of its target genes. Black arrows represent the events occurring during phosphorelay signaling.

Two-component signal transduction systems

Two-component signal transduction systems (TCSs) consist of two proteins, the response regulator (RR) and the histidine kinase (HK) (Fig 2). TCSs are found only in bacteria and some lower eukaryotes such as for instance *Dictyostelium discoideum* and *Saccharomyces cerevisiae* (9, 34). Since there is little to no homology with human components, TCSs are considered to be promising targets for therapy and prevention. The

histidine kinase is the sensor protein that resides in the cell membrane. Upon sensing and binding of its ligand (e.g., sugars or peptides), the HK is activated and autophosphorylation of a conserved histidine residue occurs. Usually, HK proteins are found as homodimers and phosphorylation of the histidine residue on the first HK catalyzes phosphorylation of the histidine residue on the second HK of the dimer (14). The second component is a cytosolic protein called the response regulator (RR), which is a DNA-binding protein. The phosphoryl group of the HK is transferred to an aspartate residue on the RR, after which the RR undergoes a conformation change and becomes activated. The activated RR then acts as a transcriptional regulator, repressing or activating gene expression.

Sequence analysis of the pneumococcal genome has revealed the presence of 13 TCSs plus one so-called orphan response regulator (RitR) (46, 60). Knock-out mutants of these genes were used to investigate their role in virulence of different serogroups in mice (30, 61). Following these two large screening studies, more detailed studies have led to further insight into the importance of pneumococcal TCSs in pathogenesis (for a complete overview see 46).

The most studied TCS in *S. pneumoniae* is the ComDE system (TCS12), which is part of the competence machinery (24). Natural competence is the state in which a bacterium is able to take up DNA from its environment. Sequestered DNA can be inserted into the chromosome, and novel genetic properties and pathogenic features can be acquired. The histidine kinase ComD senses competence stimulating peptide (CSP), a small peptide of which the precursor is produced by *comC* and is transported and cleaved to its mature state by ComAB. When CSP accumulates to a critical concentration outside the cell, ComD will be phosphorylated, and transfer of the phosphoryl group will activate the RR ComE, which in turn activates the other components of the competence machinery. The transcriptional regulator ComX is also activated, which subsequently activates many other genes involved in DNA uptake and processing, bacteriocin production, and stress response (32, 49). The ComDE TCS was demonstrated to be important for virulence, more specifically ComD was shown to be involved in pneumonia and bacteremia in serotype 2 strain D39 (3), serotype 3 strain 0100993 (31) and serotype 4 strain TIGR4 (20). Using microarray-based transcriptional profiling, over 200 CSP-responsive genes were identified (12, 49). These induced or repressed genes included several known virulence factors such as autolysin *lytA*, a stress response protein *htrA*, and the choline binding protein *cbpD*.

TCS04 was demonstrated to be involved in virulence during murine pneumonia (61), and later the involvement was shown to be strain-dependent (37). Transcriptome analysis of

mutants for *rr04* revealed variation between strains in RR04-regulated genes, potentially reflecting the need for expression of a specific set of genes during pneumococcal virulence, which varies between strains. Especially the *psa*-operon (encoding a manganese transporter) was strain-specifically regulated (37).

TCS02 (also known as *vic*, *micAB*, *yycFG*, and *492*) was found to be the only essential TCS in pneumococcus, regulating cell wall and fatty acid metabolism and the expression of the virulence factor PspA (30, 39, 43, 61). One of the important gene targets of RR02 is the *pcsB* gene encoding a murein-hydrolase; overexpression of this gene could negate the essentiality of RR02, allowing deletion of *rr02* (41, 42).

TCS05 (or CiaRH) was also shown to be important for virulence, mediated by activating the major virulence factor HtrA (22). Mutants for the response regulator CiaR displayed the same attenuation as mutants for HtrA, in a murine intranasal infection model (22). In addition, this TCS plays a role in competence and antibiotic resistance (16, 17, 64).

TCS09 was found to significantly contribute to virulence in murine models of infection (7). Mutants for the response regulator in two different strains displayed different virulence properties. D39Δ*rr09* was avirulent, whereas 0100993Δ*rr09* was fully virulent upon intraperitoneal and intravenous infection. In contrast, upon intranasal infection, this mutant was unable to survive in lung tissue and in the blood, suggesting a role in dissemination from lungs to blood (7). This study by Blue & Mitchell was one of the first to illustrate that gene regulatory systems may have different impacts on the virulence potential of different strains (7).

Nutritional gene regulation

All bacteria need nutrients to sustain and to replicate. Acquisition of these molecules or their precursors is required for both anabolic and catabolic activities of the cell. During *in vitro* growth pneumococci convert glucose into the acids acetate and/or lactate, thereby generating energy and producing molecules needed for other cellular processes. In addition, dedicated transporters exist to take up nutrients like sugars or amino acids from the environment. However, little is known about pneumococcal metabolism *in vivo* during pathogenesis, while such knowledge would be crucial to increase our understanding of pneumococcal virulence.

Gene regulatory pathways play an essential role in controlling the expression of genes necessary for uptake and consumption of particular metabolites. Various publications have reported the co-regulation of metabolism and virulence factors, and proteins involved in

metabolism are often required for virulence. For instance, in *Staphylococcus aureus* production of capsular polysaccharides and toxigenic exoproteins is repressed under carbohydrate-rich conditions, while adhesion factors are expressed (see 56 for review). Similarly, *Clostridium difficile* toxin production is associated with severe changes in metabolism (26). This co-regulation suggests that bacteria may become pathogenic when the nutritional supply decreases. By damaging the host tissue new nutrients can become available for these bacteria.

Availability of nitrogen is of high importance since this element is present in most building blocks of the bacterial cell, such as for instance DNA, RNA, and proteins. Regulation of uptake of nitrogen-containing molecules is therefore essential for the fitness of the bacterium. In many Gram-positive bacteria, CodY is one of the main transcriptional regulators responsible for controlling the activity of systems that take up or process nitrogen-containing compounds (40, 57). This protein is responsive to the presence of the branched chain amino acids (BCAAs) isoleucine, valine, and leucine. When present in high enough concentrations, BCAAs interact with CodY, which results in DNA-binding of CodY to conserved regions in the target promoters where it represses gene activity (Fig 3). In *Bacillus subtilis*, an additional molecule, GTP, modulates the repressive activity of CodY. In contrast, in lactic acid bacteria (to which pneumococci belong) it has been shown that CodY is not responsive to GTP (13). This difference might reflect the considerable physiological difference between these bacteria.

In addition to CodY, the transcriptional regulator GlnR also controls the expression of genes involved in nitrogen metabolism in *B. subtilis* (58, 59). GlnR specifically regulates glutamine/glutamate metabolism. These two amino acids form the major donors of nitrogen to the cell. GlnR controls the expression of GlnA, a metabolic enzyme converting glutamate in glutamine (58, 59). Together, CodY and GlnR form the core of nitrogen regulators in *B. subtilis* and for this reason we believe these might be important for pneumococcus as well.

Furthermore, metal cations are essential for bacterial survival. Ions such as Fe^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Mn^{2+} are often required as co-factors for enzymatic activity. In addition, these cations also differ in concentrations in the human body, where they may trigger expression of virulence factors (29, 45). Pneumococcal transcriptional regulators like SczA, RitR, and PsaR have been shown to be crucial for regulation of intracellular divalent cation concentrations and virulence (25, 28, 29, 62, 63). The zinc response regulator SczA regulates the expression of *czcD*, encoding a cation diffusion facilitator which offers resistance to high Zn^{2+} concentrations (28). In addition, the orphan response regulator RitR

(described above) regulates the expression of the iron uptake system *piuABCD* (62, 63). Finally, the manganese regulator PsaR regulates the expression of serine protease gene *prtA* and the *psa*-operon encoding the Mn^{2+} uptake lipoprotein complex PsaBCA, both involved in virulence (25, 29).

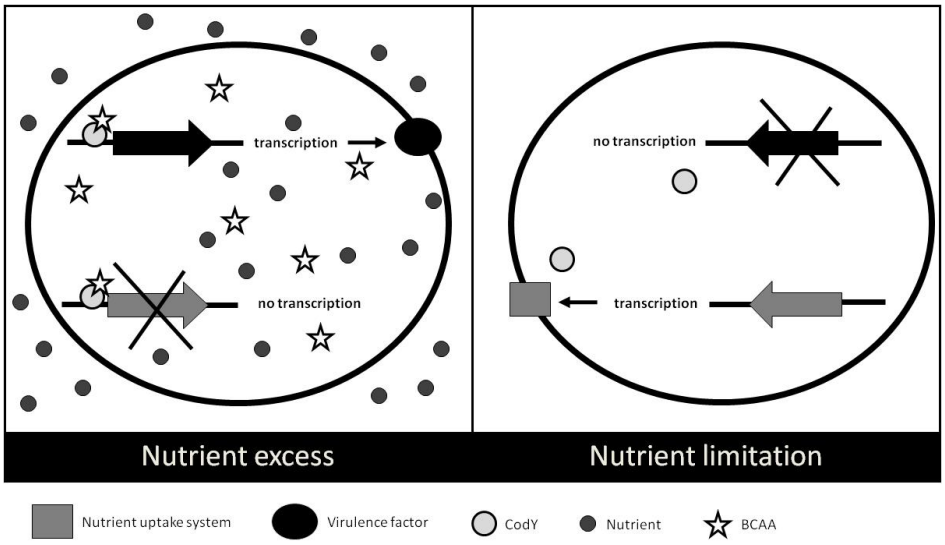

Figure 3. Model for transcriptional regulation by CodY in Gram-positive bacteria. Under nutrient excess, the bacterium has sufficient nutrients to grow and to multiply: CodY, associated with BCAAs, is repressing transcription of genes involved in catabolic processes. Hypothesized is that in this state virulence factors, such as adherence factors are activated and factors required for killing of host cells are repressed (not shown). During nutrient limitation, BCAAs become scarce and CodY will be in its inactive form, not able to repress gene transcription. Under these conditions, genes encoding nutrient uptake systems (e.g. amino acid transporters) and genes encoding catabolic enzymes will be transcribed.

Aims and outline of this thesis

Basic regulatory systems control many processes during the bacterial life cycle. Especially regulation of basic metabolism, such as nitrogen metabolism or balancing cation uptake, is essential for bacterial survival. Understanding these features, along with the factors that directly interact with host components, are essential in unraveling the processes of pneumococcal disease. Moreover, it will aid future vaccine development and innovative treatment. The studies described in this thesis focused on four different gene expression

regulatory systems, aiming to elucidate their role both in the context of pneumococcal physiology, and with respect to pneumococcal virulence.

Chapter 2 describes the role of response regulator 09 (RR09) in global gene expression and in virulence. Strain-dependent features of this regulator were identified by testing *rr09* knock out mutants in strains D39 and TIGR4 in virulence and gene expression studies. Moreover, expression levels of some of the newly identified differentially expressed genes were monitored *in vivo*.

Chapter 3 reports on the manganese-dependent transcriptional regulator PsaR. Using *psaR* knock out mutants of two strains (TIGR4 and D39), virulence studies showed strain-specific phenotypes. Transcriptome and proteome analysis revealed that transcription and translation of several genes were affected in expression by the *psaR* mutation, which differed between the two strains tested.

The nutritional regulator CodY is investigated extensively in **Chapter 4**. The identified gene regulatory pathways controlled by CodY are described, as well as the impact of CodY on virulence. Furthermore, the CodY DNA binding domain was identified, and the influence of branched chain amino acids and GTP on DNA binding by CodY was examined. Lastly, the role of *pcpA*, one of the gene targets, was investigated during adherence.

Chapter 5 and **6** describe the role of glutamine/glutamate metabolism in pneumococcus. In **Chapter 5** the genes regulated by GlnR were identified and studied at the molecular level. In addition, the DNA-binding sequence of GlnR was identified. In **Chapter 6**, the role of GlnR and its target genes described in **Chapter 5** was extended to virulence in mice.

In **Chapter 7**, we provide a comprehensive overview of the role of CodY and GlnR in metabolic regulation and virulence in pneumococci and other related species, integrating both existing literature and our own results described in **Chapters 4**, **5**, and **6**.

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CHAPTER 2

Regulation of gene expression in *Streptococcus pneumoniae* by response regulator 09 is strain dependent

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Abstract

Recent murine studies have demonstrated that the role of response regulator 09 (RR09) of *Streptococcus pneumoniae* in virulence is different in different strains. In the present study, we used a murine pneumonia model of infection to assess the virulence of a TIGR4 *rr09* mutant, and we found that TIGR4Δ*rr09* was attenuated after intranasal infection. Furthermore, we investigated the *in vitro* transcriptional changes in pneumococcal *rr09* mutants of two strains, D39 and TIGR4, by microarray analysis. The transcriptional profiles of the *rr09* mutants of both strains had clear differences compared to the profiles of the parental wild-type strains. In D39Δ*rr09*, but not in TIGR4Δ*rr09*, genes involved in competence (e.g., *comAB*) were upregulated. In TIGR4, genes located on the *rlrA* pathogenicity islet, which are not present in the D39 genome, appeared to be regulated by RR09. Furthermore, several phosphotransferase systems (PTSs) believed to be involved in sugar uptake (e.g., the PTS encoded by *sp0060* to *sp0066*) were strongly downregulated in D39Δ*rr09*, while they were not regulated by RR09 in TIGR4. To examine the role of one of these PTSs in virulence, D39Δ*sp0063* was constructed and tested in a murine infection model. No difference between the virulence of this strain and the virulence of the wild type was found, indicating that downregulation of the *sp0063* gene alone is not the cause of the avirulent phenotype of D39Δ*rr09*. Finally, expression of *rr09* and expression of three of our identified RR09 targets during infection in mice were assessed. This *in vivo* experiment confirmed that there were differences between expression in wild-type strain TIGR4 and expression in the *rr09* mutant, as well as differences between expression in wild-type strain D39 and expression in wild-type strain TIGR4. In conclusion, our results indicate that there is strain-specific regulation of pneumococcal gene expression by RR09.

Introduction

Streptococcus pneumoniae (pneumococcus) is a common asymptomatic commensal of the nasopharynx in healthy individuals. However, in young children, in the elderly, and in immunocompromised people, this Gram-positive bacterium is a major cause of otitis media, pneumonia, and septicemia. To persist at these various sites, the pneumococcus needs to adapt and orchestrate its gene expression. Two-component signal transduction systems (TCSs) play a central role in bacterial survival by regulating various cellular processes, such as osmoregulation, sporulation, genetic competence, and chemotaxis, in response to environmental changes (1, 27). TCSs typically consist of a membrane-associated sensory protein called a histidine kinase (HK) and a cognate cytosolic DNA-binding response regulator (RR), which acts as a transcriptional regulator. When an external signal is sensed, a histidine residue of the histidine kinase autophosphorylates, after which this phosphogroup is transferred to the response regulator. This results in a conformational change in the regulatory protein, which can then perform its regulatory function.

The pneumococcal genome contains 13 putative complete TCSs plus one orphan response regulator (15, 28). Ten of the pneumococcal TCSs have been shown to be important for virulence (for a review, see reference 22). For instance, CiaR/CiaH has been demonstrated to contribute to virulence (25, 29), probably in part through control of expression of *htrA*, which encodes a serine protease that is a major virulence factor (11, 19). Recently, TCS06 has been found to regulate the expression of *cbpA*, which codes for a major adhesin that is also a protective antigen (26).

Relatively little is known about TCS09, which consists of an RR encoded by sp0661 (spr0578 in the R6 genome) and an HK encoded by sp0662 (spr0579). Amino acid sequence homology data suggest that the sensory domain of the HK is related to the extracellular part of McpA and McpB of *Bacillus subtilis* (15). These proteins are believed to be involved in the control of chemotaxis through sensing of environmental nutrient concentrations. The extracellular stimulus of TCS09, however, is not known.

Several in vivo studies have demonstrated that RR09 has a role in virulence. Interestingly, the contribution of RR09 to virulence varies with the bacterial strain and the site of infection (3, 6, 15, 29). It has been suggested that in strain 0100993 (serotype 3) RR09 is involved in the dissemination from the lung to the systemic circulation, since attenuation of 0100993Δ*rr09* was observed in a murine pneumonia model but not in a sepsis model of infection (3). In D39 (serotype 2) the lack of RR09 led to an avirulent phenotype upon intranasal, intravenous, or intraperitoneal infection (3). The genetic differences between

strains are likely to have a significant impact on the repertoire of genes regulated by RR09. In line with this, TCS04 mutants were recently found to confer strain-dependent phenotypes in a murine infection model, possibly caused by differential regulation of pneumococcal surface antigen A (20).

So far, no gene targets of RR09 that could account for the observed in vivo phenotypes of *rr09* mutants have been identified, although sequence homology data have suggested that TCS09 is involved in nutrient perception (3, 15). To investigate strain differences in the role of TCS09 in pneumococcal virulence further, we examined the phenotype of a TIGR4 *rr09* mutant using a murine pneumonia model. In addition, we used DNA microarray technology to examine how transcriptional patterns are affected by a lack of *rr09*. To this end, global expression profiles of wild-type and *rr09* mutants were analyzed during different stages of in vitro growth. We assessed strain-specific regulation by RR09 by comparing transcriptional profiles of mutants with two different genetic backgrounds, namely, strains D39 and TIGR4. In vitro expression experiments and data analysis were performed independently in two laboratories (Rotterdam and Glasgow) using slightly different methods, after which the data were validated and combined. Finally, we assessed expression of *rr09* and three of its identified in vitro targets during experimental virulence in mice.

Materials and Methods

Pneumococcal strains

Wild-type *S. pneumoniae* strains D39 (= NCTC 7466; serotype 2) and TIGR4 (= ATCC BAA-334; serotype 4) were used in this study. The Δrr09 derivative of TIGR4 was constructed by insertional inactivation using an erythromycin resistance cassette, as described previously for D39Δrr09 (3). Pneumococcal strains were grown on Columbia blood base agar supplemented with 5% (vol/vol) defibrinated sheep blood (and supplemented with 1 μg/ml erythromycin for the rr09 mutant strains). For RNA isolation analyses, cultures were grown in Todd-Hewitt broth supplemented with 5 g/liter yeast extract (THY broth) or in brain heart infusion broth (BHI broth) without erythromycin until they reached the desired turbidity. For construction of the sp0063 mutant (annotated spr0062 in R6), approximately 100-bp portions of up- and downstream regions of the targeted sequence were amplified by PCR using primers 50L (TCTATGATTGGTATTTCTATCGTAGG) and 50M (GGCGCGCCTGAGGTAAGATCATGTAAAGGTAACC) and primers 50N (TCTTACCTCAGGCGCGCCACTGCCTTTATCTTCTGGTTGCTTGG) and 50O (CAAATTTAGCAGTAAATTCTTCTGGG), respectively. These fragments were joined by overlap extension PCR due to overlap in the 50 M and 50N primers. This procedure also introduced an AscI site between the upstream and downstream sequences via the primers. The fusion PCR product was cloned into TOPO-pCR4 (Invitrogen) and confirmed by sequencing. The spectinomycin resistance cassette from pDL278 was cloned into the AscI site, and the resulting construct was used for transformation of D39. Spectinomycin-resistant colonies were verified by PCR. This mutation resulted in replacement of nucleotides 176 to 676 of sp0063 with the spectinomycin resistance cassette.

Mice and infections

Female outbred MF1 mice (body weight, 25 to 30 g) were purchased from Harlan Olac, Bicester, United Kingdom. For pneumonia infection, 9-week-old mice were lightly anesthetized with 2.5% (vol/vol) halothane, after which an infection dose consisting of 1.0 x 10^6 CFU of wild-type strain TIGR4 and the Δ*rr09* mutant resuspended in 50 μl of sterile phosphate-buffered saline (PBS) was administered in the nostrils of mice held vertically (12). At predetermined times after infection, groups of mice were sacrificed by cervical dislocation, and blood samples were removed by cardiac puncture using a 1-ml syringe. Bronchoalveolar lung lavage and sampling of the lungs were performed as described previously (13). The

viable bacteria in lung and blood samples were counted by plating serial 10-fold dilutions on blood agar base no. 2 (Oxoid, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (13). Mice infected with wild-type strain D39 and the Δ*sp0063* mutant were inoculated intraperitoneally with 100 µl PBS containing 1 x 10^6 CFU.

Survival times were analyzed using the Mann-Whitney U test. The bacteriology results are expressed below as geometric means ± standard errors of the means. Bacterial loads obtained in the time experiment were compared using Student's *t* test. In all analyses, a *P* value of <0.01 was considered statistically significant.

RNA extraction

Five hundred milliliters of THY broth was inoculated with 10 to 20 colonies from agar plates, and the cultures were statically grown at 37°C. Samples for RNA isolation were removed when the cultures reached an optical density at 600 nm (OD_{600}) of either 0.1 (early log phase) or 0.2 (mid-log phase), and the pneumococcal cells were harvested by centrifugation for 10 min at 3,300 x *g* and 4°C. Subsequently, the cells were resuspended in 400 µl of THY broth supplemented with 1.5 g of glass beads (diameter, 0.1 mm; Sigma), 500 µl of phenol, 50 µl of 10% (wt/vol) sodium dodecyl sulfate (SDS), and 50 µl of 3 M sodium acetate (pH 5.2). Each mixture was then snap-frozen in liquid nitrogen and stored at –80°C until it was used. The cells were lysed by vigorous shaking for 8 min at 4°C and subsequently centrifuged for 10 min at 9,300 x *g* and 4°C. The upper phase was mixed with an equal volume of chloroform and centrifuged for 2 min at 16,000 x *g* and 4°C. After this, RNA was purified using a High Pure RNA isolation kit (Roche Diagnostics). Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Roche Diagnostics). RNA was isolated from three replicate cultures of D39 and TIGR4.

Bacterial RNA isolated at an OD_{600} of 0.6 (mid-log phase) was obtained from two independent cultures grown in BHI broth at 37°C. Bacteria were lysed three times for 20 s in the presence of 200 µl of lysozyme (15 mg/ml) and 50 mg of glass beads (100 µm; Sigma) using a Hybaid Ribolyser (Hybaid) set at speed 4. Subsequently, RNA was isolated using RNeasy Midi columns (QIAGEN) with an on-column DNase digestion step.

cDNA labeling

Synthesis and subsequent labeling of cDNA for microarray hybridization were performed essentially as described previously (31). Briefly, 20 µg of total RNA was incubated for 16 h at 42°C in the presence of 400 U Superscript III RNase H^- reverse transcriptase

(Invitrogen), 0.2 mM aminoallyl dUTP (Amersham), 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.3 mM dTTP, and 3.2 µg random nonamers. The synthesized cDNA was then labeled by coupling either Cy3 or Cy5 to the dUTP aminoallyl reactive group (CyScribe postlabeling kit; Amersham Biosciences) and was purified using a GFX PCR, DNA, and gel band purification kit (Amersham Biosciences).

Microarray construction

Two different DNA microarray platforms were used for analysis of samples isolated at low and high OD_{600}; these platforms are referred to below as array 1 for an OD_{600} of 0.1 or 0.2 and array 2 for an OD_{600} of 0.6. Array 1 was constructed as described previously (14, 31) and contained amplicons representing 2,087 open reading frames (ORFs) of *S. pneumoniae* TIGR4 and 184 unique *S. pneumoniae* R6 ORFs, all spotted in duplicate.

Array 2 was designed at the Pathogen Functional Genomics Resource Centre at TIGR (http://www.pfgrc.tigr.org/). The complete genome array consisted of amplicons representing segments of 2,131 open reading frames of *S. pneumoniae* reference strain TIGR4 spotted in quadruplicate on glass slides. In addition, the array contained 563 open reading frames amplified from strains R6 (164 ORFs) and G54 (399 ORFs).

Microarray hybridization

For array 1, labeled wild-type and Δ*rr09* cDNA were combined and dried using a SpeedVac. The cDNA was dissolved in 10 µl of Slidehyb #1 hybridization buffer (Ambion Europe Ltd.), boiled for 3 min, and kept on ice until hybridization. Prewarmed (68°C) Slidehyb #1 hybridization buffer was added to obtain a final hybridization volume of 60 µl, after which the sample was applied to a prewarmed array and incubated in a hybridization incubator (ISO20; Grant) overnight at 42°C. Slides were removed from the incubator and washed with 2x sodium chloride-sodium citrate buffer (SSC) containing 0.5% (wt/vol) SDS for 5 min, followed by two washes in 1x SSC containing 0.25% SDS and in 1x SSC containing 0.1% SDS for 5 min each. Subsequently, the slides were dried by centrifugation. In all cases, dye swapping was performed with one of the three biological replicates.

For array 2, denatured labeled wild-type and Δ*rr09* probes were mixed and dried using a SpeedVac (Savant DNA 110 SpeedVac; Global Medical Instruments). The labeled probes were dissolved in 30 µl of filtered hybridization buffer (50% formamide, 5x SSC, 0.1% SDS, 300 µg salmon sperm DNA) and heated to 95°C for 5 min. The samples were applied to glass slides and incubated for 18 h at 42°C in a GeneChip 640 hybridization oven (Affymetrix).

After hybridization, the slides were washed in 2x SSC-0.1% SDS for 5 min at 55°C, and this was followed by two washes in 0.1x SSC-0.1% SDS for 5 min and in 0.1x SSC for 5 min. For each independent RNA sample one dye swap was performed.

Microarray data analysis

For array 1, dual-channel array images were acquired with a GeneTac LS IV confocal laser scanner (Genomics Solutions) and were analyzed with the ArrayPro 4.5 software (Media Cybernetics Inc.). Spots were screened visually to identify the low-quality spots. Slide data were processed using MicroPreP as described previously (4, 31, 32). Prior to analysis, automatically and manually flagged spots and spots with very low background-subtracted signal intensities (5% of the weakest spots [sum of Cy3 and Cy5 net signals]) were filtered out of all data sets. Net signal intensities were calculated using grid-based background subtraction. In Prep, a grid-based Lowess transformation was performed for slide normalization, with an *f* value (percentage of the spots used for curve fitting) of 0.5 and an nSteps value (number of iterations) of 5. In Postprep, negative and empty values were removed, and outliers were removed by the deviation test. Further analysis was performed using a Cyber-T Student's *t* test for paired data (18). This web-based program lists the ratios of all intrareplicates (duplicate spots) and interreplicates (different slides), the mean ratios per gene, and standard deviations and *P* values assigned to these mean ratios. For identification of differentially expressed genes, only genes with a minimum of three reliable measurements (i.e., data from at least two different slides) and a *P* value of <0.05 were included. Since this *P* value is purely a statistical measure of differential gene expression and reproducibility across replicates, an additional change cutoff of twofold was applied.

Hybridized array 2 images were acquired with a Perkin-Elmer Scan Array Express. The spot intensities were defined and quantified using BlueFuse for Microarray 3.1 (BlueGnome Ltd.). The data were analyzed further with GeneSpring 7.0 (Silicon Genetics). Lowess intensity-dependent normalization was used to perform per-spot and per-array normalization, and the cross-gene error model was based on the replicate measurements. Statistically significant differences were defined as differences with a Student's *t* test *P* value of <0.05 and a ratio change threshold of at least 2 standard deviations over the median ratio for each strain.

Isolation of pneumococcal RNA during experimental infection

Female outbred CD-1 mice (body weight, 20 to 30 g) were purchased from Harlan Olac, Bicester, United Kingdom. Nine-week-old mice were lightly anesthetized with 2.5% (vol/vol) halothane, after which 1.0 x 10^7 CFU wild-type D39, wild-type TIGR4, or TIGR4Δ*rr09* resuspended in 50 µl of sterile PBS was administered in the nostrils of mice held vertically. Control mice were inoculated with sterile PBS alone. Twenty-four hours postinfection, mice were sacrificed by cervical dislocation, and nasopharyngeal lavage fluid, bronchoalveolar lavage fluid (BALF), lungs, and blood were collected. After collection of 2 ml nasopharyngeal lavage fluid or BALF, 20 µl was used for determination of the bacterial load, and the remaining fluid was mixed with 4 ml RNAprotect (QIAGEN) and incubated for 5 min at room temperature. Bacteria were collected by centrifugation (16,000 x *g*, 5 min, 4°C), and the pellets were snap-frozen in liquid nitrogen. After collection of blood, 20 µl was used for determination of the bacterial load, and the remaining blood was added to 5 ml RNAprotect (QIAGEN). To separate pneumococci from host mouse cells, the mixtures were centrifuged for 10 min at 825 x *g* and 4°C. Each supernatant was transferred to a new tube and centrifuged for 5 min at 16,000 x *g* and 4°C. The pelleted bacteria were snap-frozen in liquid nitrogen. The lungs that were collected were homogenized in 2 ml RNAprotect. The lung samples were handled as described above for the blood samples. RNA of all samples was isolated using an RNeasy kit (QIAGEN) with on-column DNase treatment (QIAGEN). Subsequently, RNA isolated from homogenized lungs and blood were enriched for bacterial RNA using a MicrobEnrich kit (Ambion). Finally, all RNA samples were amplified using a SenseAmp kit (Genisphere). Pneumococcal gene expression was measured in samples obtained from three individual mice.

Real-time PCR

Real-time PCR was used to validate the microarray data (in vitro) and to investigate expression of several genes during experimental virulence in mice (in vivo). DNA-free total RNA (2.5 µg) was reverse transcribed using 1 µg (for the in vitro experiments with array 1) and 0.5 µg (for the in vivo experiments) of random hexamers and Superscript III reverse transcriptase (Invitrogen). To confirm the absence of genomic DNA, reactions without reverse transcriptase were performed. Subsequently, 1 µl of cDNA diluted 1:10 (for in vitro experiments) or 1:4 (for in vivo experiments) was used as the template in real-time PCR. Duplicate quantitative PCR assays were performed using a DyNAmo HS SYBR green quantitative PCR kit (Bioke) with an ABI Prism 7700 according to the manufacturer's

instructions. Primers (sequences are available on request) were designed using the Oligo 6.22 software (Molecular Biology Insights) and were used at a concentration of 300 nM for 40 cycles of amplification (15 s at 95°C and 1 min at 60°C), which was followed by a melting curve analysis to verify the product homogeneity.

Validation of selected targets from array 2 was performed in essentially the same manner, with a few minor differences. Two micrograms of RNA was reverse transcribed using 6 μg of random hexamers and Superscript III reverse transcriptase (Invitrogen). Serial dilutions of cDNA were used as templates in real-time PCR assays with a DNA Engine Opticon 2 (MJ Research), using the following reaction conditions: 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The relative quantitation method (17) was used to evaluate the quantitative variation between wild-type and Δ*rr09* strains for each gene examined. The *gyrA* (sp1219) amplicon was used as an internal control for normalization of data.

Cyber-T website

The Cyber-T website is http://visitor.ics.uci.edu/genex/cybert/index.shtml.

Accession numbers

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession numbers GSE6137 (array 1) and GSE6139 (array 2).

Results and Discussion

In vivo characteristics of TIGR4Δ*rr09*

Previous studies indicated that RR09 has a strain-specific role in pneumococcal virulence, as D39 with deletion of the response regulator *rr09* was found to be avirulent in all murine models tested, while 0100993Δ*rr09* was found to be attenuated only upon intranasal infection (3). Here, we extended these observations by analyzing a TIGR4Δ*rr09* mutant in a murine pneumonia model of infection. Mice infected intranasally with 10^6 CFU of TIGR4Δ*rr09* were found to have significantly longer survival times than mice infected with the parental strain (Fig. 1a). None of the mice infected with the wild-type strain survived longer than 30 h after inoculation. The level of survival of mice infected with Δ*rr09* dropped to 20% after 48 h, and none of these mice survived longer than 100 h after inoculation. Furthermore, the TIGR4Δ*rr09* bacterial counts in BALF, lungs, and blood showed that there were significant reductions compared to the wild-type strain ($P < 0.01$) (Fig. 1b). This indicated that like 0100993Δ*rr09*, the TIGR4Δ*rr09* mutant was attenuated upon intranasal infection but was not avirulent like D39Δ*rr09*.

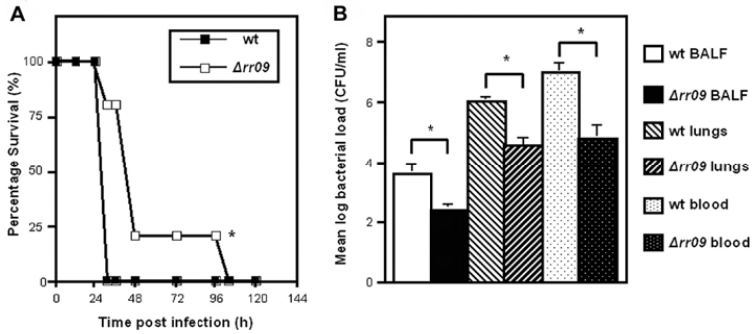

Figure 1. (A) Survival of mice after intranasal infection with 10^6 CFU TIGR4. The asterisk indicates that the *P* value is <0.01 for a comparison of the survival times of TIGR4Δ*rr09* and wild-type strain TIGR4. **(B)** Bacterial loads in BALF, homogenized lungs, and blood 24 h after intranasal infection with 10^6 CFU. An asterisk indicates that the *P* value is <0.01 for a comparison of the bacterial loads recovered from different sites. The bars indicate geometric means, and the error bars indicate standard errors of the means. wt, wild type.

Microarray analysis of pneumococcal mutants

To identify genes controlled by RR09, we compared the transcriptional profiles of *S. pneumoniae* wild-type strains D39 and TIGR4 and their isogenic mutant Δ*rr09* derivatives at

various stages of growth. During this analysis, we used two different amplicon-based microarrays, both designed to provide nonredundant representation of the sequenced R6 and TIGR4 genomes. Importantly, no differences in *in vitro* growth between the wild-type strains and the Δ*rr09* mutants were detected, indicating that the observed differential gene expression was not a result of altered growth rates.

Comparison of D39 and TIGR4

Little overlap was observed in the sets of genes controlled by RR09 in D39 and TIGR4, with a few notable exceptions (Fig. 2a and b and Table 1). The levels of transcription of two genes, encoding a putative lactose phosphotransferase system (PTS) repressor (*sp0875*) and a 1-phosphofructokinase (*sp0876*), were increased in both D39 and TIGR4 Δ*rr09* mutants in BHI broth at an OD_{600} of 0.6 (Fig. 2a and Table 1). Five genes were downregulated in both Δ*rr09* derivatives (Fig. 2b and Table 1). Five genes appeared to be oppositely regulated by RR09 in D39 and TIGR4 when they were grown in THY broth; these genes included *glnR* and *pyrR*, which were upregulated in D39Δ*rr09* and downregulated in TIGR4 when the organisms were grown in THY broth (Table 1).

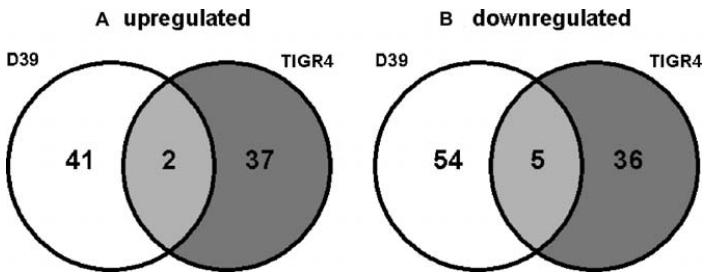

Figure 2. Distribution of genes regulated by RR09 in D39 and TIGR. **(A)** Upregulation in the Δ*rr09* mutants; **(B)** downregulation in the Δ*rr09* mutants.

Table 1. Genes regulated by RR09 in both D39 and TIGR4[a].

Gene	Annotation	Ratios[b]					
		D39			TIGR4		
		OD_{600} =					
		0.1	0.2	0.6	0.1	0.2	0.6
Commonly regulated							
sp0875	Lactose phosphotransferase system repressor, *lacR*	–0.06	–0.51	**3.93**	–0.44	–0.09	**1.46**
sp0876	1-Phosphofructokinase, putative	–0.12	–0.33	**3.74**	–0.40	–0.08	**1.37**
sp0647	PTS, galactitol-specific IIC, putative	**–2.40**	**–2.48**	NA[c]	0.11	–0.17	**–1.11**
sp0648	Beta-galactosidase, *bgaA*	**–3.26**	**–3.47**	NA	–0.02	0.82	**–1.80**
sp1804	General stress protein 24, putative	**–1.05**	–0.66	NA	–1.39	–0.55	**–1.04**
sp1883	Dextran glucosidase *dexS*, putative	**–1.55**	**–2.47**	NA	–0.26	0.28	**–1.45**
sp1884	Trehalose PTS, IIABC components	**–1.19**	**–1.50**	NA	0.03	0.11	**–1.87**
Oppositely regulated							
sp0090	ABC transporter, permease protein	NA	0.36	**–2.01**	**2.38**	0.21	NA
sp0501	Transcriptional regulator, *glnR*	NA	NA	**1.51**	–0.10	**–1.06**	NA
sp0964	Dihydroorotate dehydrogenase B, *pyrD*	0.82	**1.36**	NA	–1.00	**–1.05**	NA
sp1278	Pyrimidine operon regulatory protein, *pyrR*	**1.45**	**1.5**	NA	**–2.93**	**–1.22**	NA
sp2141	Glycosyl hydrolase-related protein	–0.05	0.01	**–3.62**	**1.96**	0.17	NA

[a] Commonly regulated genes showed the same expression pattern in D39 and TIGR4, while oppositely regulated genes were found to be upregulated in the *rr09* mutant in one strain and downregulated in the other.

[b] The values are $\log_2$(expression in Δ*rr09* mutant/expression in wild type) values. The values in bold type are the values for genes that are considered to be differentially expressed.

[c] NA, no valid data were acquired.

Gene regulation by RR09: D39

A total of 102 genes were found to be differentially expressed in wild-type strain D39 and the Δ*rr09* mutant. Forty-three of these genes could be considered to be directly or indirectly repressed by RR09 as they were upregulated in the D39 Δ*rr09* derivative compared to the expression in the wild type (Fig. 3a; see Tables S1 to S3 in the supplemental material, page 50-54).

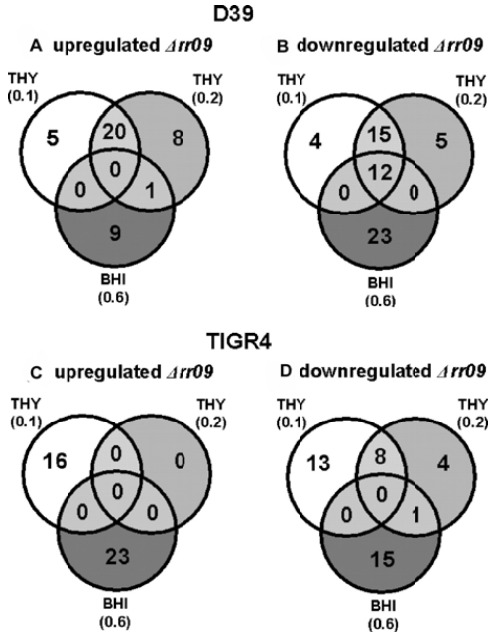

Figure 3. Distribution of genes regulated by RR09 at OD_{600} of 0.1 and 0.2 in THY broth and at an OD_{600} of 0.6 in BHI broth. **(A)** Genes upregulated in D39Δ*rr09*; **(B)** genes downregulated in D39Δ*rr09*; **(C)** genes upregulated in TIGR4Δ*rr09*; **(D)** genes downregulated in TIGR4Δ*rr09*.

The transcription of genes involved in genetic competence, such as *comAB*, *comDE*, *cinA*, *comX*, and *cglABCD*, increased significantly in D39Δrr09 in the early and mid-log phases of growth in THY broth. Upregulation of *comDE* induces competence, which in turn induces transcription of *comX*. Peterson et al. described genes induced by competence-stimulating peptide and described three groups of responding genes: early, late, and delayed genes (24). In this study, upregulation of early genes (regulated by ComE) and late genes (*cglABCD* and *cinA*) may have been the result of premature development of competence caused by the lack of RR09.

A similar pattern of increased expression in the *rr09* mutant was observed for genes encoding products predicted to be involved in pyrimidine and purine synthesis (e.g., *sp1277* and *sp1278*), riboflavin kinase (*sp1110*), and a polypeptide deformylase (*sp1549*). Interestingly, the polypeptide deformylase has been suggested to be a broad-range antimicrobial target (5). In BHI broth at an OD_{600} of 0.6, the notable genes showing increased expression in the Δ*rr09* mutant included *glnAR*, encoding glutamate synthetase and its repressor, and a putative operon encoding a lactose phosphotransferase system repressor, 1-

phosphofructokinase, and a fructose-specific PTS (*sp0875* to *sp0877*). Finally, expression of a putative operon containing the pneumolysin gene, as well as several hypothetical ORFs (*sp1923* to *sp1926*), was found to be upregulated in D39Δ*rr09* only at an OD_{600} of 0.6.

The levels of transcription of 59 genes were significantly decreased in D39Δ*rr09*, meaning that these genes are directly or indirectly activated by RR09 (Fig. 3b; see Tables S1 to S3 in the supplemental material). These genes include a putative operon (sp2141 to sp2144) predicted to be involved in *N*-glycan degradation and the serine protease *htrA* gene (10, 11, 25), which are downregulated at an OD_{600} of 0.6 in BHI broth.

The set of genes downregulated in D39Δ*rr09* was found to be enriched for genes predicted to encode proteins involved in carbohydrate metabolism, particularly genes encoding enzyme II of nine sugar-specific phosphotransferase systems. These membrane-associated proteins facilitate the uptake of carbohydrates such as mannose and fructose. For instance, several genes encoding the putative mannose-specific PTS IIABC (*sp0061* to *sp0064*), the cellobiose-specific PTS IIABC (*sp0305* to *sp0310*), and the galactitol-specific PTS IIABC (*sp0645* to *sp0647*) were in this group. However, genes encoding other components of these phosphotransferase systems, including enzyme I, HPr, and CcpA (global regulator of carbon metabolism) (for a review, see reference 30), did not appear to be regulated by RR09 in D39. Expression of these genes may be controlled by a different transcriptional regulator.

Gene regulation by RR09: TIGR4

In TIGR4, there were significant differences in expression of 80 genes, and there was hardly any overlap between the growth phases sampled, underscoring the importance of sampling at multiple times. Thirty-nine genes were upregulated in TIGR4Δ*rr09* (Fig. 3c; see Tables S4 to S6 in the supplemental material), including genes encoding the iron compound ABC transporter (*piuD* [*sp1871*]) and five hypothetical proteins expressed early during growth (OD_{600} = 0.1). At the highest cell density, increased expression of a whole cluster of genes predicted to be involved in purine metabolism (*sp0044* to *sp0056*) was observed in the mutant.

Several of the 41 genes found to be repressed in TIGRΔ*rr09* are predicted to encode products involved in metabolism of (amino) sugars and purines or pyrimidines (e.g., *sp0266* and *sp1278*), particularly during early growth in THY broth (Fig. 3d; see Tables S4 to S6 in the supplemental material). Later during growth in BHI broth, the downregulated genes included the gene encoding pneumococcal surface protein A (*pspA*), *clpL*, coding for the

ATP-dependent ClpL protease, and various stress response genes (*hrcA*, *grpE*, *dnaK*, and *dnaJ*), as well as genes in an ABC transporter operon (sp1895 to sp1897).

An interesting pattern of regulation was observed for genes located on the *rlrA* pathogenicity islet: the genes were strongly downregulated in THY broth but strongly upregulated in BHI broth. This 12-kb islet, which is not present in the D39 genome (2, 23, 28), encodes the transcriptional activator RlrA, three surface proteins (RrgA, RrgB, and RrgC), and three putative sortases (SrtB, SrtC, and SrtD). RlrA has been shown to positively regulate the expression of these seven genes on the pathogenicity islet, including itself (7). While repression of the pathogenicity islet by another regulator, MgrA, has been reported (8), no differential expression of *mgrA* was observed in this study, possibly due to a low level of expression and hence low signals. The pathogenicity islet has been shown to contribute to virulence (2, 6, 7), and thus, unbalanced regulation in TIGR4Δ*rr09* may have resulted in the attenuation that we observed in the murine model (Fig. 1). Although the regulatory mechanism of this pattern of expression remains unclear, the differences in regulation between the two broth media used suggest that there is indirect regulation, which might involve a number of downstream regulators controlled by RR09 in a growth-phase-dependent manner.

Real-time PCR analysis

To validate our microarray data, relative transcript levels were determined by quantitative real-time PCR for a selection of genes for all growth phases sampled. Overall, the expression ratios obtained by microarray and real-time PCR analyses were found to be strongly positively correlated for both D39 ($R^2 = 0.86$; $n = 24$) and TIGR4 ($R^2 = 0.90$; $n = 31$). For example, expression of *comA* was confirmed to be strongly upregulated in D39 Δrr09, and the *comA* expression levels in TIGR4 Δrr09 and the wild-type strain were found to be similar by both methods (not shown).

An interesting target of RR09 identified by our microarray analysis was the *rlrA* pathogenicity islet of TIGR4, which had an unusual, growth-phase-dependent pattern of expression. Real-time PCR analysis of two genes of this locus, *rrgA* and *rlrA*, confirmed this transcriptional profile: there was strong downregulation at the lower optical densities, and there was strong upregulation later (Fig. 4). In addition, we validated the levels of expression of other genes (*htrA*, *spoJ*, *pspA*, *sp1896*, and *sp2141* to *sp2144*) identified as differentially expressed by microarray analysis and found that there was agreement between the microarray and real-time PCR results (not shown).

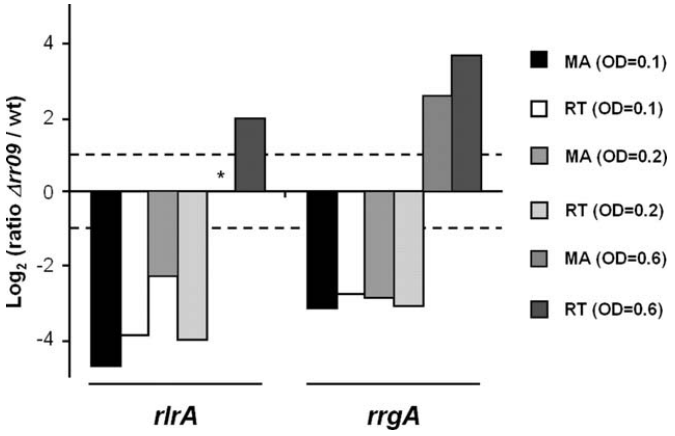

Figure 4. Expression of *rlrA* and *rrgA* in TIGR4. Log_2(Δ*rr09*/wild type) was determined by microarray analysis (MA) or real-time PCR (RT). The dashed lines indicate the twofold change microarray cutoff values for differential expression. The asterisk indicates that no microarray data were obtained for *rlrA* at an OD_{600} of 0.6. wt, wild type.

Expression of pneumococcal genes during experimental virulence

To determine to what extent the observed in vitro gene regulation by RR09 reflected the in vivo situation, we examined expression of *rr09* and three putative targets during experimental infection of mice with D39, TIGR4, and TIGR4Δ*rr09*. Due to its avirulent phenotype, analysis of gene expression in D39Δ*rr09* during infection was not possible. To correct for potential differences in the bacterial load or RNA yield, the levels of expression of sampled genes were normalized to the level of expression of *gyrA*. The levels of expression of the housekeeping gene *ddl* (*sp1671*) did not vary much in all of the strains in the compartments sampled (Fig. 5). The *rr09* transcript could be detected in mice infected with both wild-type D39 and TIGR4, indicating that *rr09* is indeed expressed in vivo. With the exception of the nasopharynx, the levels of *rr09* expression were comparable for the two strains in all compartments sampled (Fig. 5a). Strikingly, the level of *rr09* expression was higher in the lumen of the lungs (BALF) than in the other compartments sampled (not shown). The *in vivo* expression levels of putative RR09 targets appeared to be different in wild-type D39 and TIGR4. For example, the levels *comA* of expression were higher in D39 than in TIGR4 (Fig. 5a). Differential *in vivo* gene expression in wild-type strain TIGR4 and TIGR4Δ*rr09* was also observed. The level of expression of *comA* was lower in TIGR4Δ*rr09*

than in wild-type strain TIGR4 in BALF, in contrast to the in vitro expression data (in THY broth), which indicated that there was no regulation of this gene by RR09 in TIGR4.

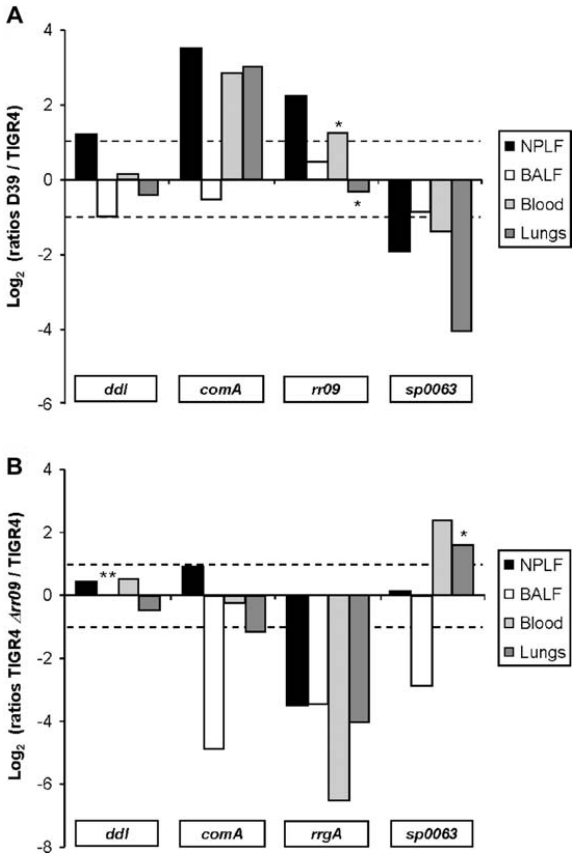

Figure 5. Expression of pneumococcal genes during experimental infection measured by real-time PCR. Log_2 ratios of wild-type strain D39/wild-type strain TIGR4 **(A)** and TIGR4Δ*rr09*/wild-type strain TIGR4 **(B)** are the averages of expression measured in three individual mice; the only exceptions were when two mice were used (indicated by one asterisk) and when no valid data were acquired (indicated by two asterisks). NPLF, nasopharyngeal lavage fluid.

The levels of expression of the *rrgA* gene, encoding a structural unit of the pilus encoded by the *rlrA* pathogenity islet (2, 16), were also determined during experimental virulence, and this gene found to be expressed in all compartments sampled in the TIGR4 wild-type strain. The levels of expression of *rrgA* in TIGR4Δ*rr09* were found to be 10- to 90-fold lower than the levels of expression in the TIGR4 wild-type strain (Fig. 5b). This finding

correlated well with our in vitro microarray and real-time PCR data, supporting the hypothesis that RR09 has a role in the regulation of this pathogenicity islet.

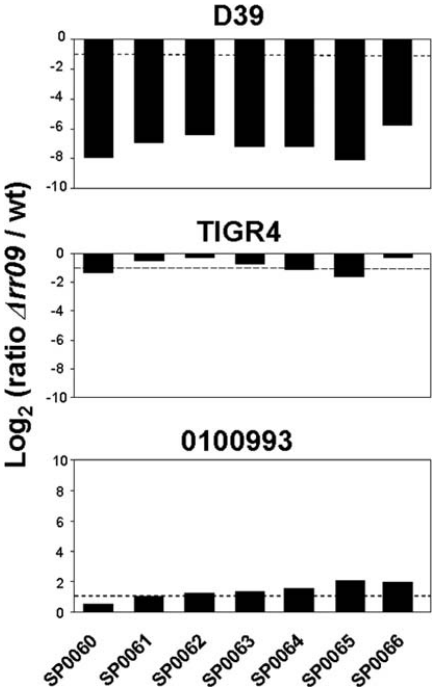

Figure 6. Expression of *sp0060* to *sp0066* in wild-type and Δ*rr09* mutant D39, TIGR4, and 0100993 as determined by real-time PCR. The dashed line indicates the twofold change microarray cutoff value for differential expression. wt, wild type.

Strain-dependent regulation of *sp0063* does not explain the observed difference in virulence

While our analysis indicated that RR09 has a prominent role in regulation of sugar metabolism in D39, only three putative PTSs were found to be regulated by RR09 in TIGR4. It seems unlikely that there is a difference in the sugar diets of D39 and TIGR4, as both genomes basically contain the same putative PTSs (20 PTSs common to both strains and one PTS specific for TIGR4) (9, 28). A clear example of a PTS locus differentially regulated by RR09 in D39 and TIGR4 was the locus comprising *sp0060* to *sp0066*, and therefore we also examined expression of this locus by real-time PCR in a third strain, 0100993. This and previous studies showed that RR09 mutants of the three strains used here all have different phenotypes in murine models of infection (2). While strong downregulation of *sp0060* to

sp0066 was confirmed to occur in D39Δ*rr09*, no RR09-dependent regulation was observed in either TIGR4 or 0100993 (Fig. 6). The observed downregulation of this locus in D39Δ*rr09* may result in reduced sugar uptake and thus a smaller supply of carbohydrates for the polysaccharide capsule, which in turn might explain the avirulent phenotype of D39Δ*rr09*. To test this hypothesis, an *sp0063* mutant was constructed and tested in a murine model, and it was compared with the D39 wild-type strain and the *rr09* mutant. There was no significant difference in the survival rates between wild-type strain D39- and *sp0063* mutant-infected mice; all five wild-type strain-infected mice died, as did four of the five Δ*sp0063*-infected mice. Likewise, the blood counts at 24 h postinfection were similar (Fig. 7). In agreement with our previous work (3), the *rr09* mutant was essentially avirulent; all infected mice survived, and the blood counts at 24 h were below the detection limit (~83 CFU/ml). Indeed, the mice showed no overt clinical signs of infection. Thus, the gene product of *sp0063* alone does not significantly contribute to virulence in D39 in this model and hence does not explain the dramatic phenotype seen after deletion of *rr09* in D39. The Δ*rr09* derivatives of TIGR4 and 0100993 conferred a less severe *in vivo* phenotype. *In vivo* expression of *sp0063* could be demonstrated for both D39 and TIGR, and the levels in TIGR4 were higher (almost fourfold) (Fig. 5a). In TIGR4, *sp0063* appears to be regulated by RR09 *in vivo* (Fig. 5b) but not *in vitro* (Fig. 6), highlighting the importance of measuring gene expression *in vivo*. Moreover, this finding provides further evidence that the differential regulation of this operon observed *in vitro* does not explain the difference between D39 and TIGR4 *in vivo*. Also, there appears to be a redundancy of PTS in the pneumococcal genome, which could complement the lower level of expression of the PTS. This is underscored by our preliminary data, which indicate that the *rr09* and *sp0063* mutants are able to ferment mannose (not shown).

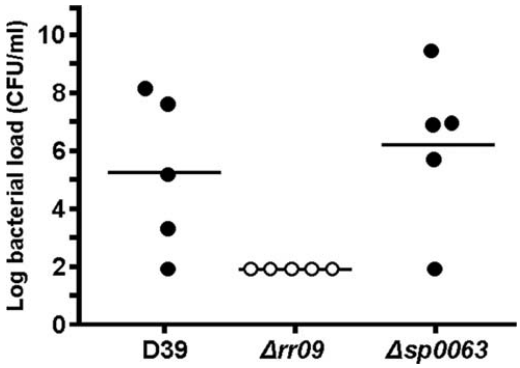

Figure 7. Bacterial loads in blood of mice 24 h after intraperitoneal infection with 10^6 CFU. No differences between D39Δ*sp0063* and the wild-type strain were observed; in contrast, in the Δ*rr09* strain the bacterial loads were below the detection limit ($\log_{10}$ 1.92) and were assigned a value of 1.90.

In conclusion, we identified several targets of RR09 in D39 and TIGR4 that could account for the phenotypes of their mutants, and we demonstrated that *rr09* and three of its targets are expressed *in vivo*. These targets include both common and strain-specific targets of RR09. During *in vitro* growth, some genes were found to be controlled by RR09 at all phases of growth examined, while other genes appeared to be regulated in a growth-phase-dependent manner. Although this differential expression could have been the result of the different growth conditions and/or of the use of two different microarrays, this explanation appears less likely considering the considerable overlap between the sets of genes regulated at the different growth phases (e.g., *sp0060* to *sp0066*, *sp0645* to *sp0648*, and the *rlrA* pathogenicity island). The expression of pneumococcal genes in different broth media has been shown to differ substantially (21), but our results show that apparent putative targets can be identified with different experimental setups. The targets identified can be either directly or indirectly regulated by RR09. Further experiments, such as DNA binding assays, are necessary to distinguish between these two possibilities. DNase footprinting can be used to identify the RR09 DNA-binding sequence, and in order to obtain an initial view of the RR09 regulon in other strains, the consensus binding sequence could subsequently be used for *in silico* screening. Although the exact roles of most of the identified targets in pneumococcal virulence still have to be investigated, a few targets have already been studied extensively. Furthermore, predictions of the functions of many of the novel RR09 targets identified here are still based on sequence homology alone, and further studies are required to identify the exact roles in pneumococcal virulence. Pneumococci are likely to have different nutritional

needs during the various stages of infection. Possibly, RR09 plays a role in this process by regulating genes required for nutrient uptake in response to various conditions characteristic of different sites in the host. The signal that triggers RR09 might be present at one site and not at another, and particular strains might respond differently to these signals, potentially explaining the phenotype differences observed in animal studies.

Acknowledgments

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Supplemental material

Table S1. Genes up- and downregulated in D39 *Δrr09* at an optical density of 0.1 grown in THY-broth. Ratios are given as *Δrr09* / wild type.

Gene	Annotation	Ratio (fold change)	*p*-value
Upregulated			
SP0042	competence factor transporting protein *comA*	4.88	2.14E-04
SP0043	competence factor transport protein *comB*	5.00	2.60E-03
SP0430	hypothetical protein	2.50	8.67E-03
SP0545	immunity protein *blpY*	2.05	1.47E-02
SP0963	dihydroorotate dehydrogenase *pyrDII*	2.06	1.82E-03
SP1110	riboflavin kinase / FMN adenylyltransferase, *mreA*	4.01	2.36E-04
SP1266	DNA processing protein *dprA*, putative	2.16	3.55E-02
SP1277	aspartate carbamoyltransferase catalytic chain, *pyrB*	4.19	2.49E-02
SP1278	pyrimidine operon regulatory protein, *pyrR*	2.73	5.75E-04
SP1286	uracil permease, *pyrP*	2.17	4.28E-02
SP1549	polypeptide deformylase, *def*	2.66	1.17E-02
SP1716	ABC transporter permease - Na+ export, *natB*	5.99	1.88E-02
SP1717	ABC transporter permease - Na+ export, *natA*	2.29	1.53E-06
SP1941	competence/damage-inducible protein, *cinA*	2.46	1.26E-04
SP2006	transcriptional regulator, *comX*	10.84	3.74E-02
SP2047	hypothetical protein	2.33	2.82E-02
SP2048	competence protein *comGF*	3.17	2.20E-03
SP2050	competence protein *cglD*	2.87	5.36E-03
SP2051	competence protein *cglC*	4.53	2.20E-02
SP2052	competence protein *cglB*	3.74	4.07E-04
SP2053	competence protein *cglA*	8.25	8.94E-04
SP2156	SPFH domain/band 7 family	2.05	9.86E-04
SP2235	response regulator *comE*	11.29	1.50E-04
SP2236	sensor histidine kinase *comD*	15.54	2.67E-04
SPXX57	SP1908; single strand binding protein, *ssb*	2.61	2.68E-03
Downregulated			
SP0060	beta-galactosidase, *bgaA*	0.16	1.51E-04
SP0061	PTS system, mannose-specific IIB component, putative	0.33	6.50E-04
SP0062	PTS system, mannose-specific IIC component, putative	0.22	2.19E-03
SP0063	PTS system, mannose-specific IID component, putative	0.16	2.48E-02
SP0064	PTS system, mannose-specific IIA component, putative	0.30	9.47E-04
SP0065	sugar isomerase domain protein, *agaS*	0.35	2.40E-02
SP0066	aldose 1-epimerase, *galM*	0.35	5.68E-03
SP0303	6-phospho-beta-glucosidase, *bglA*	0.08	1.97E-04
SP0306	transcriptional regulator *bglG*, putative	0.04	1.84E-03
SP0307	PTS system, IIA component	0.09	5.91E-03
SP0308	PTS system, cellobiose-specific IIA component	0.09	1.40E-02
SP0309	hypothetical protein	0.25	7.67E-04
SP0310	PTS system, cellobiose-specific IIC component	0.14	6.37E-04
SP0626	branched-chain amino acid transport system II carrier protein, *brnQ*	0.34	1.76E-02
SP0645	PTS system, galactitol-specific IIA component, putative	0.29	3.13E-03
SP0646	PTS system, galactitol-specific IIA component, putative	0.29	2.25E-03

SP0647	PTS system, galactitol-specific IIC component, putative	0.19	1.47E-04
SP0648	beta-galactosidase, *bgaA*	0.10	2.42E-03
SP0782	hypothetical protein	0.38	5.20E-03
SP0923	Cof family protein	0.49	3.68E-02
SP1190	tagatose 1,6-diphosphate aldolase, *lacD*	0.15	8.23E-05
SP1191	tagatose-6-phosphate kinase, *lacC*	0.12	7.43E-06
SP1192	galactose-6-phosphate isomerase, *lacB*	0.03	1.19E-02
SP1193	galactose-6-phosphate isomerase, *lacA*	0.14	1.57E-04
SP1197	PTS system, galactitol-specific IIB component, putative	0.16	6.20E-03
SP1804	general stress protein 24, putative	0.48	3.88E-05
SP1845	exodeoxyribonuclease, *exoA*	0.45	3.91E-02
SP1883	dextran glucosidase *dexS*, putative	0.34	7.58E-05
SP1884	trehalose PTS system, IIABC components	0.44	7.00E-05
SP2108	maltose/maltodextrin ABC transporter, *malX*	0.36	6.10E-04
SP2110	maltodextrin ABC transporter, permease protein, *malD*	0.49	1.46E-02

Table S2. Genes up- and downregulated in D39 *Δrr09* at an optical density of 0.2 grown in THY-broth. Ratios are given as *Δrr09* / wild type.

Gene	Annotation	Ratio (fold change)	*p*-value
Upregulated			
SP0018	competence protein, *comW*	22.08	3.44E-02
SP0042	competence factor transporting protein *comA*	6.56	3.19E-03
SP0043	competence factor transport protein *comB*	6.12	5.65E-04
SP0430	hypothetical protein	2.42	2.56E-02
SP0502	glutamine synthetase, *glnA*	4.59	2.26E-02
SP0545	immunity protein *blpY*	3.49	1.05E-03
SP0701	orotidine 5'-phosphate decarboxylase, *pyrF*	2.73	2.75E-03
SP0702	orotate phosphoribosyltransferase *pyrE*	2.82	3.19E-04
SP0963	dihydroorotate dehydrogenase *pyrDII*	3.41	9.85E-04
SP0964	dihydroorotate dehydrogenase B, *pyrD*	2.56	7.03E-03
SP0965	endo-beta-N-acetylglucosaminidase, *lytB*	3.11	2.07E-02
SP1110	riboflavin kinase / FMN adenylyltransferase, *mreA*	6.74	3.87E-05
SP1277	aspartate carbamoyltransferase catalytic chain, *pyrB*	2.14	2.50E-05
SP1278	pyrimidine operon regulatory protein, *pyrR*	2.83	1.10E-04
SP1380	putative ABC-2 type transport system permease	2.25	3.22E-02
SP1549	polypeptide deformylase, *def*	3.08	2.06E-02
SP1716	ABC transporter permease - Na+ export, *natB*	2.58	5.84E-06
SP1717	ABC transporter permease - Na+ export, *natA*	2.57	1.14E-04
SP1870	ABC transporter membrane-spanning permease, *fatC*	3.95	3.19E-02
SP1941	competence/damage-inducible protein, *cinA*	2.78	2.31E-03
SP2047	hypothetical protein	2.51	3.08E-04
SP2048	competence protein *comGF*	2.69	7.91E-04
SP2050	competence protein *cglD*	2.34	3.54E-03
SP2051	competence protein *cglC*	2.65	2.87E-02
SP2052	competence protein *cglB*	2.09	5.20E-05
SP2053	competence protein *cglA*	3.75	2.33E-04
SP2222	CDP-diacylglycerol-glycerol-3-p 3-phosphatidyltransferase, *pgsA*	2.23	2.24E-02
SP2235	response regulator *comE*	10.09	3.79E-04
SP2236	sensor histidine kinase *comD*	22.01	1.49E-05
Downregulated			
SP0060	beta-galactosidase, *bgaA*	0.10	5.97E-04
SP0061	PTS system, mannose-specific IIB component, putative	0.27	2.19E-03
SP0062	PTS system, mannose-specific IIC component, putative	0.19	2.86E-03
SP0063	PTS system, mannose-specific IID component, putative	0.18	6.47E-04
SP0064	PTS system, mannose-specific IIA component, putative	0.36	4.66E-04
SP0065	sugar isomerase domain protein, *agaS*	0.16	8.04E-05
SP0066	aldose 1-epimerase, *galM*	0.10	2.61E-03
SP0303	6-phospho-beta-glucosidase, *bglA*	0.03	2.25E-05
SP0306	transcriptional regulator *bglG*, putative	0.01	2.77E-03
SP0307	PTS system, IIA component	0.01	1.05E-04
SP0308	PTS system, cellobiose-specific IIA component	0.01	3.95E-04
SP0309	hypothetical protein	0.10	1.49E-03
SP0310	PTS system, cellobiose-specific IIC component	0.08	2.01E-04
SP0342	glucan 1,6-alpha-glucosidase, *dexB*	0.48	4.02E-03
SP0645	PTS system, galactitol-specific IIA component, putative	0.13	5.17E-06

SP0646	PTS system, galactitol-specific IIA component, putative	0.07	1.05E-05
SP0647	PTS system, galactitol-specific IIC component, putative	0.18	2.14E-04
SP0648	beta-galactosidase, *bgaA*	0.09	5.41E-04
SP0758	PTS system, glucose-specific IIABC components, *ptsG*	0.30	4.30E-03
SP0782	hypothetical protein	0.38	1.10E-04
SP1182	lactose phosphotransferase system repressor, *lacR*	0.47	9.21E-04
SP1190	tagatose 1,6-diphosphate aldolase, *lacD*	0.06	1.01E-03
SP1191	tagatose-6-phosphate kinase, *lacC*	0.08	3.71E-05
SP1192	galactose-6-phosphate isomerase, *lacB*	0.05	1.32E-04
SP1193	galactose-6-phosphate isomerase, *lacA*	0.03	2.26E-03
SP1197	PTS system, galactitol-specific IIB component, putative	0.09	1.70E-04
SP1626	30S ribosomal protein S15, *rpsO*	0.31	2.58E-02
SP1883	dextran glucosidase *dexS*, putative	0.18	7.71E-03
SP1884	trehalose PTS system, IIABC components	0.35	3.84E-05
SP2108	maltose/maltodextrin ABC transporter, *malX*	0.35	5.14E-04
SP2110	maltodextrin ABC transporter, permease protein, *malD*	0.31	6.30E-03
SPXX36	SP2063; LysM domain protein (**spr1875**)	0.24	1.39E-02

Table S3. Genes up- and downregulated in D39 *Δrr09* at an optical density of 0.6 grown in BHI-broth. Ratios are given as *Δrr09* / wild type.

Gene	Annotation	Ratio (fold change)	*p*-value
Upregulated			
SP0501	transcriptional regulator, MerR family	2.85	1.08E-02
SP0502	glutamine synthetase, type I	3.02	1.54E-03
SP0875	lactose phosphotransferase system repressor	15.26	2.70E-04
SP0876	1-phosphofructokinase, putative	13.35	2.36E-05
SP0877	PTS system, fructose specific IIABC components	8.35	2.72E-03
SP1922	conserved hypothetical protein	3.42	1.56E-03
SP1923	pneumolysin	4.60	2.41E-03
SP1924	hypothetical protein	2.92	3.00E-03
SP1925	hypothetical protein	3.09	4.14E-03
SP1926	hypothetical protein	2.90	2.32E-04
Downregulated			
SP0057	beta-N-acetylhexosaminidase	0.17	1.21E-03
SP0060	beta-galactosidase	0.03	7.48E-05
SP0061	PTS system, IIB component	0.07	9.99E-05
SP0062	PTS system, IIC component	0.03	1.03E-04
SP0063	PTS system, IID component	0.03	7.25E-05
SP0064	PTS system, IIA component	0.04	1.14E-04
SP0065	sugar isomerase domain protein AgaS	0.20	2.23E-02
SP0066	aldose 1-epimerase	0.12	2.97E-04
SP0090	ABC transporter, permease protein	0.25	6.19E-03
SP0285	alcohol dehydrogenase, zinc-containing	0.36	2.61E-03
SP0303	6-phospho-beta-glucosidase	0.04	8.93E-05
SP0305	PTS system, IIB component	0.09	7.50E-04
SP0306	transcriptional regulator, putative	0.21	3.50E-03
SP0307	PTS system, IIA component	0.30	2.35E-02
SP0308	PTS system, IIA component	0.25	4.82E-03
SP0310	PTS system, IIC component	0.16	1.62E-04
SP0368	Cell wall surface anchor family protein	0.22	6.38E-04
SP0386	sensor histidine kinase, putative	0.41	1.60E-02
SP0498	endo-beta-N-acetylglucosaminidase, putative	0.19	5.05E-04
SP0577	PTS system, beta-glucosides-specific IIABC	0.27	9.38E-03
SP1027	conserved hypothetical protein	0.25	1.37E-02
SP1471	oxidoreductase, putative	0.33	6.98E-03
SP1695	acetyl xylan esterase, putative	0.23	1.41E-03
SP1802	hypothetical protein	0.19	2.12E-03
SP2026	alcohol dehydrogenase, iron-containing	0.25	2.78E-03
SP2055	alcohol dehydrogenase, zinc-containing	0.35	1.87E-04
SP2056	N-acetylglucosamine-6-phosphate deacetylase	0.34	8.35E-04
SP2107	4-alpha-glucanotransferase	0.27	3.81E-03
SP2141	glycosyl hydrolase-related protein	0.08	7.90E-04
SP2142	ROK family protein	0.08	5.19E-04
SP2143	conserved hypothetical protein	0.16	2.36E-03
SP2144	conserved hypothetical protein	0.09	3.47E-04
SP2146	conserved hypothetical protein	0.20	2.06E-03
SP2239	serine protease	0.18	7.39E-04
SP2240	spspoJ protein	0.21	5.54E-03

Table S4. Genes up- and downregulated in TIGR4 *Δrr09* at an optical density of 0.1 grown in THY-broth. Ratios are given as *Δrr09* / wild type.

Gene	Annotation	Ratio (fold change)	*p*-value
Upregulated			
SP0089	hypothetical protein	2.42	3.90E-02
SP0090	ABC transporter, permease protein	5.20	4.37E-02
SP0394	PTS system, mannitol-specific IIBC components, *mtlA*	5.48	2.84E-02
SP0397	mannitol-1-phosphate 5-dehydrogenase	79.70	4.93E-03
SP0504	hypothetical protein	2.40	1.37E-02
SP0784	glutathione reductase, *gor*	2.04	2.03E-02
SP0978	competence protein *coiA*	41.15	5.63E-03
SP1058	hypothetical protein	4.05	1.72E-02
SP1059	hypothetical protein	2.20	1.06E-03
SP1774	transcriptional regulator, putative	2.14	4.45E-02
SP1828	UDP-glucose 4-epimerase, *galE*	2.79	4.92E-02
SP1832	hypothetical protein	2.07	3.32E-02
SP1871	iron-compound ABC transporter, ATP-binding protein, *piuD*	2.72	2.00E-02
SP1969	type II DNA modification methyltransferase, putative	2.37	1.24E-02
SP2141	glycosyl hydrolase-related protein	3.89	2.58E-02
SPXX18	SP0368; cell wall surface anchor family protein (spr0328)	4.66	8.91E-03
Downregulated			
SP0156	response regulator 07	0.02	4.40E-02
SP0216	50S ribosomal protein L16, *rplP*	0.30	3.78E-02
SP0261	undecaprenyl diphosphate synthase, *uppS*	0.30	4.74E-02
SP0266	glucosamine--fructose-6-phosphate aminotransferase, *glmS*	0.08	9.26E-03
SP0294	50S ribosomal protein L13, *rplM*	0.16	4.26E-02
SP0461	transcriptional regulator, *rlrA*	0.03	1.54E-03
SP0462	cell wall surface anchor family protein, *rrgA*	0.13	2.50E-03
SP0463	cell wall surface anchor family protein, *rrgB*	0.13	5.22E-03
SP0464	cell wall surface anchor family protein, *rrgC*	0.06	2.54E-03
SP0467	sortase *srtC*, putative	0.20	6.92E-04
SP0468	sortase *srtD,* putative	0.11	1.74E-02
SP0623	dipeptidase, *pepV*	0.31	4.95E-02
SP0751	branched-chain amino acid ABC transporter, *livM*	0.05	2.45E-02
SPXX56	SP0857; ABC transporter, *aliA*-like	0.33	4.60E-02
SP0858	hypothetical protein	0.28	3.27E-02
SP1023	acetyltransferase, GNAT family	0.46	2.18E-03
SP1278	pyrimidine operon regulatory protein, *pyrR*	0.13	9.37E-03
SP1805	hypothetical protein	0.48	1.14E-02
SP2005	hypothetical protein	0.47	9.31E-03
SP2094	hypothetical protein	0.39	3.25E-02
SP2202	hypothetical protein	0.01	1.32E-02

Table S5. Genes up- and downregulated in TIGR4 *Δrr09* at an optical density of 0.2 grown in THY-broth. Ratios are given as *Δrr09* / wild type.

Gene	Annotation	Ratio (fold change)	*p*-value
Upregulated			
none			
Downregulated			
SP0117	pneumococcal surface protein A, *pspA*	0.47	4.37E-06
SP0266	glucosamine--fructose-6-phosphate aminotransferase, *glmS*	0.37	4.35E-02
SP0461	transcriptional regulator, *rlrA*	0.20	3.80E-03
SP0462	cell wall surface anchor family protein, *rrgA*	0.10	6.33E-03
SP0463	cell wall surface anchor family protein, *rrgB*	0.23	1.77E-03
SP0464	cell wall surface anchor family protein, *rrgC*	0.19	8.29E-03
SP0467	sortase *srtC*, putative	0.18	2.58E-02
SP0468	sortase *srtD*, putative	0.44	3.00E-03
SP0501	transcriptional regulator, *glnR*	0.48	7.29E-06
SP0661	response regulator 09	0.49	5.61E-03
SP0964	dihydroorotate dehydrogenase B, *pyrD*	0.48	3.22E-02
SP1278	pyrimidine operon regulatory protein, *pyrR*	0.43	2.46E-03
SP1965	hypothetical protein	0.43	2.56E-02

Table S6. Genes up- and downregulated in TIGR4 *Δrr09* at an optical density of 0.6 grown in BHI-broth. Ratios are given as *Δrr09* / wild type.

Gene	Annotation	Ratio (fold change)	*p*-value
Upregulated			
SP0044	phosphoribosylaminoimidazole-succinocarboxamide synthase	5.29	2.30E-04
SP0045	phosphoribosylformylglycinamidine synthase, putative	6.10	1.02E-04
SP0046	amidophosphoribosyltransferase	13.52	1.30E-04
SP0047	phosphoribosylformylglycinamide cyclo-ligase	8.18	2.21E-04
SP0048	phosphoribosylglycinamide formyltransferase	8.07	1.44E-04
SP0050	phosphoribosylaminoimidazolecarboxamide formyltransferase	7.51	5.00E-05
SP0051	phosphoribosylamine--glycine ligase	7.85	1.07E-05
SP0053	phosphoribosylaminoimidazole carboxylase, catalytic subunit	7.60	2.03E-05
SP0054	phosphoribosylaminoimidazole carboxylase, ATPase subunit	11.82	2.81E-05
SP0056	adenylosuccinate lyase	3.10	3.59E-05
SP0287	xanthine/uracil permease family protein	7.66	7.24E-05
SP0288	conserved hypothetical protein	3.21	4.35E-04
SP0289	dihydropteroate synthase	2.19	5.46E-04
SP0462	cell wall surface anchor family protein	5.87	3.16E-04
SP0463	cell wall surface anchor family protein	7.54	2.29E-04
SP0464	cell wall surface anchor family protein	6.67	3.70E-04
SP0466	sortase, putative	2.87	4.60E-04
SP0845	lipoprotein	2.60	1.94E-04
SP0875	lactose phosphotransferase system repressor	2.75	8.13E-05
SP0876	1-phosphofructokinase, putative	2.59	2.61E-04
SP1229	formate--tetrahydrofolate ligase	3.23	6.25E-04
SP1249	conserved hypothetical protein	2.55	5.77E-04
SP1587	oxalate:formate antiporter	6.93	3.33E-05
Downregulated			
SP0117	pneumococcal surface protein A	0.40	5.25E-04
SP0338	ATP-dependent Clp protease, ATP-binding subunit, putative	0.37	1.31E-02
SP0424	(3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase	0.46	1.50E-03
SP0515	heat-inducible transcription repressor HrcA	0.41	4.40E-04
SP0516	heat shock protein GrpE	0.32	8.74E-04
SP0517	dnaK protein	0.40	1.21E-03
SP0519	dnaJ protein	0.49	5.98E-04
SP0647	PTS system, IIC component, putative	0.46	4.36E-03
SP0648	beta-galactosidase	0.29	4.47E-04
SP0724	hydroxyethylthiazole kinase, putative	0.47	1.12E-04
SP1804	general stress protein 24, putative	0.49	1.55E-02
SP1883	dextran glucosidase DexS, putative	0.37	1.46E-02
SP1884	trehalose PTS system, IIABC components	0.27	2.07E-03
SP1895	sugar ABC transporter, permease protein	0.41	2.30E-03
SP1896	sugar ABC transporter, permease protein	0.45	1.48E-03
SP1897	sugar ABC transporter, sugar-binding protein	0.37	1.34E-03

CHAPTER 3

Strain-specific impact on PsaR of *Streptococcus pneumoniae* on global gene expression and virulence

PsaR and pneumococcal virulence

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Abstract

Previous studies have indicated that PsaR of *Streptococcus pneumoniae* is a manganese-dependent regulator, negatively affecting the expression of at least seven genes. Here, we extended these observations by transcriptome and proteome analysis of *psaR* mutants in strains D39 and TIGR4. The microarray analysis identified three shared PsaR targets: the *psa*-operon, *pcpA*, and *prtA*. Additionally, we found 31 genes to be regulated by PsaR in D39 only, most strikingly a cellobiose-specific PTS and a putative bacteriocin operon (*sp0142-sp0146*). In TIGR4, 14 PsaR gene targets were detected, with the *rlrA* pathogenicity islet being the most pronounced. Proteomics confirmed most of the shared gene targets. To examine the contribution of PsaR to pneumococcal virulence, we compared D39 and TIGR4 wild-type (wt) and *psaR*-mutants in three murine infection models. During colonization, no clear effect was observed of the *psaR* mutation in either D39 or TIGR4. In the pneumonia model, small but significant differences were observed in the lungs of mice infected with either D39wt or Δ*psaR*: D39Δ*psaR* had an initial advantage in survival in the lungs. Conversely, TIGR4Δ*psaR*-infected mice had significantly lower bacterial loads at 24h only. Finally, during experimental bacteremia, D39Δ*psaR*-infected mice had significantly lower bacterial loads in the blood stream than wt-infected mice for the first 24h of infection. TIGR4Δ*psaR* showed attenuation at 36h only. In conclusion, our results show that PsaR of D39 and TIGR4 has a strain-specific role in global gene expression and in the development of bacteremia in mice.

Introduction

Streptococcus pneumoniae encounters different environments during its life cycle: in most cases it inhabits the human nasopharynx where it resides asymptomatically, but it can also spread through the body, causing severe infections (5). What exactly triggers the pneumococcus to cause infections at these sites in the body is poorly understood. In response to environmental changes, the transcriptional program is likely to change, which is considered to be niche-specific and can result in expression of distinct virulence factors.

Manganese ions (Mn^{2+}) are important for bacterial life, for instance serving as cofactors for metalloenzymes. The function of these metalloenzymes is widespread among bacterial cellular processes, such as glycolysis, gluconeogenesis, and oxidative stress defense (26). In pneumococcus, involvement of Mn^{2+} in competence has also been described (12), but most studies have focused on its involvement in oxidative stress (25, 28, 39, 40, 42, 48). In the latter process, Mn^{2+} serves as a cofactor for superoxide dismutase, an important enzyme that provides defense against superoxide radicals (3). As the concentration of manganese is much higher in saliva than in blood plasma (9), fluctuations in the amount of this trace element might serve as a trigger for expression of certain virulence factors.

Various transcriptional regulators have been described for the pneumococcus (20) and several large scale mutagenesis studies have identified a role in virulence for these regulators (8, 18, 21, 36, 43). One of them is encoded by *psaR*, a transcriptional regulator responsive to Mn^{2+} and negatively affecting the expression of the *psa* operon, *pcpA*, *rlrA*, and *prtA* (28, 32). Recently, the PsaR-binding sequence was identified in the promoter region of *psaBCA*, *prtA*, and *pcpA*, and a genome-wide screen for this binding sequence did not show any other putative targets (31). Moreover, it has been reported that regulation by PsaR is opposite in reaction to two cations, namely repression in high Mn^{2+} and derepression in high Zn^{2+} (32).

The *pcpA* gene encodes a choline binding protein (46) and *rlrA* encodes a transcriptional regulator, which controls the expression of the *rlrA* pathogenicity islet (19). The *psaBCA* operon encodes the Psa permease that transports the cations Mn^{2+} and Zn^{2+} into the cell (12, 28, 29, 37, 39). The permease has been implicated in virulence and protection against pneumococcal infection (6, 47). Moreover, PsaA has been shown to bind to E-cadherin, a surface molecule of the host cells (2, 44, 45). PsaR homologues are found in other streptococci, where they regulate homologues of *psaBCA* and contribute to virulence (17, 27, 41). The role of PsaR in pneumococcal virulence has been investigated in an EF3030 (serotype 19F) genetic background (28). During seven days of colonization no difference

between the wild-type and its isogenic *psaR*-mutant was observed, while after 7 days of lung infection, the *psaR*-mutant was significantly attenuated compared to the wild-type (28).

In this study, we examined the effect of the deletion of *psaR* on global gene and protein expression in two other pneumococcal strains, D39 (serotype 2) and TIGR4 (serotype 4) using transcriptomics and proteomics. Furthermore, we investigated the contribution of PsaR to pneumococcal virulence in three murine infection models representing the major phases in the life cycle of *S. pneumoniae*: colonization, pneumonia, and bacteremia.

Materials and Methods

Bacterial strains and media

The pneumococcal strains used in this study are listed in Table 1 and were grown at 37˚C in Todd Hewitt Yeast broth (THY), in chemically defined medium (CDM, recipe available on request), or on Colombia base agar (Oxoid) supplemented with 5% sheep blood (Biotrading). Pneumococcal strains were maintained in 10% (v/v) glycerol, 10% skim milk at -80˚C. *Escherichia coli* DH5α (Stratagene) was grown in Luria Broth at 37˚C while shaking or on Luria Broth agar. Media were supplemented with antibiotics (50 mg/l ampicillin and/or 20 mg/l trimethoprim) when appropriate.

Table 1. Pneumococcal strains used in this study.

Strain	Gene Identifier	Antibiotic resistance	Reference
D39 wild-type	-	-	NCTC 7466; serotype 2
D39Δ*psaR*	*spd1450*	trimethoprim	This study
TIGR4 wild-type	-	-	ATCC BAA-334; serotype 4
TIGR4Δ*psaR*	*sp1638*	trimethoprim	This study

Construction of *psaR*-mutants

The gene encoding *psaR* was deleted from strain TIGR4 (*sp1638*) and D39 (*spd1450*) by allelic replacement with the *dfr13* cassette conferring trimethoprim resistance (1). To this end, *psaR* with 1,000 bp of upstream and downstream flanking sequences was amplified from chromosomal TIGR4 DNA using primer pair psaRSacFw and psaRKnpRv (all primers are listed in Table 2). This amplicon was cloned into pBlueScript KS+ (Stratagene) using the SacII and KpnI restriction sites. The coding sequence of *psaR* was deleted from the plasmid by performing an inverse PCR with primer pair psaRNotFw and psaRSalRv, amplifying the *psaR*-flanking sequences and pBlueScript KS+ and introducing NotI and SalI restriction sites for further cloning. This amplicon was ligated to the *dfr13*-cassette, which was amplified from pKOT (22) with the primers TmpSalFw and TmpNotRv, to create the knockout construct pKO*psaR*-T4, and transformed to *E. coli* DH5α. A 2,620-bp linear DNA fragment containing *psaR*-flanking DNA and *dfr13* was amplified from pKO*psaR*-T4 using primer pair psaRSacFw and psaRKnpRv. This PCR product was used to delete *psaR* from the genome of

S. pneumoniae TIGR4 by CSP-2-induced (100 ng/ml) transformation. Transformants were selected on the basis of trimethoprim-resistance and were checked by sequencing for recombination at the desired location on the chromosome, i.e., replacement of *psaR* by *dfr3* (which will be transcribed in the opposite direction of *psaR*). Wild-type TIGR4 was subsequently transformed with chromosomal DNA isolated from these *ΔpsaR* transformants to rule out the possibility of any additional mutations on the chromosome. The identical procedure was performed for the construction of D39Δ*psaR*, with the exception that the 3' chromosomal region from TIGR4 of pKO*psaR*-T4 was replaced by the D39-specific 3' *psaR*-region. This region differs between the two strains: it contains an ISS element in TIGR4, and a small ORF encoding a unique hypothetical protein in D39. Transformation of D39 was induced with CSP-1 (100ng/ml).

Table 2. Oligonucleotide primers used in this study.

Primername	Sequence (5'-3')[a]	Restriction site	Strain
psaRSacFw	GCGCCCGCGGGGAATTTGCATCCTCTTCTCC	*SacII*	D39/TIGR4
psaRKnpRv	GCGCGGTACCATATTGCCCATCAGCTTTCC	*KpnI*	TIGR4
psaRNotFw	GCGCGCGGCCGCTCCTCAGTAACGACGAGGATTT	*NotI*	D39/TIGR4
psaRSalRv	GCGCGTCGACGCAGGTCTATGCCAATTTCA	*SalI*	D39/TIGR4
TmpSalFw	CGCGGTGGTCGACGGATTTTTGTGAGCTTGGACT	*SalI*	D39/TIGR4
TmpNotRv	GGGGGGCCGCGGCCGCTTACGACGCGCATAGACG	*NotI*	D39/TIGR4
psaRKpnRv-D39	GAAAATGGTACCAGAGAGCAAGAGCCACTC	*KpnI*	D39
SeqTmpFw	ATAAATGCGGACCGATTCC	-	D39/TIGR4
SeqTmpRv	GCCTTCTTCCCAGTGCTTAAC	-	D39/TIGR4

[a] Restriction sites on oligonucleotide primers are underlined

Transcriptional profiling of D39 and TIGR4 psaR-mutants

Microarray analysis was performed essentially as described (22, 24). In short, 500 ml of CDM was inoculated with 10-20 colonies from agar plates, and these cultures were statically grown at 37°C. Samples for RNA isolation were taken when the cultures reached an optical density (OD_{600}) of 0.2 (mid-log growth). RNA was isolated and purified using the High Pure RNA isolation kit (Roche diagnostics) as described (22, 24). Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Roche diagnostics). RNA was isolated from three replicate cultures. Synthesis, subsequent labeling of cDNA, and microarray hybridization was performed as described (24, 33). In all cases, dye-swapping was performed with one of the three biological replicates. Microarrays used in this study were constructed as described (24, 33) and contain amplicons representing 2,087 ORFs of S. pneumoniae TIGR4 and 184 ORFs unique for S. pneumoniae R6, all spotted in duplicate.

DNA microarray data analysis

Dual-channel array images were acquired with a GeneTac LS IV confocal laser scanner (Genomics Solutions) and analyzed with ArrayPro 4.5 software (Media Cybernetics Inc.). Spots were screened visually to identify those of low quality. Slide data were processed using MicroPreP as described (13, 24, 49). Prior to analysis, automatically and manually flagged spots and spots with very low background-subtracted signal intensity (5% of the weakest spots (sum of Cy3 and Cy5 net signals)), were filtered out of all datasets. Spots with a signal in one channel and no signal in the other were subjected to an empty-value assignment of 1%, after which net signal intensities were calculated using a grid-based background subtraction. A grid-based Lowess transformation was performed for slide normalization, negative and empty values were removed, and outliers were removed by the deviation test. Further analysis was performed using a Cyber-T Student's *t* test for paired data (38). For identification of differentially expressed genes, only genes with a minimum of six reliable measurements, a Bayesian *P*-value < 0.001, a False Discovery Rate (FDR) < 0.05, and a standard deviation < ratio were included. Since these criteria are purely a statistical measure of differential gene expression and reproducibility across replicates, an additional fold-change cut-off of 2 was applied.

Stable isotope labeling in cell culture (SILAC)

For SILAC experiments D39 wild-type and *psaR*-mutant strains were inoculated in THY and grown to mid-log phase. These cultures were used to inoculate CDM, supplemented with both lysine and arginine as the light ($^{12}C_6$ L-lysine, $^{12}C_6$ L-arginine; *psaR*-mutant) or heavy ($^{13}C_6$ L-lysine, $^{13}C_6$ L-arginine; wild-type) isotopic counterparts. When these cultures reached an OD_{600} of 0.2, they were diluted to an OD_{600} of 0.04 in fresh pre-warmed CDM with the appropriate heavy or light lysine and arginine, and grown to an OD_{600} of 0.2. This was repeated until the cells were grown in heavy or light lysine and arginine-containing CDM for at least 5-6 generations. After the last generation, the cells were harvested by centrifugation and washed once with PBS. Equal amounts of heavy-labeled wild-type and light-labeled mutant cells were combined and used for mass spectrometry. Bacterial pellets were lysed in lysis buffer (7 M urea, 2 M thiourea, protease inhibitor mix (Roche), pH 8.0). Lysates were subjected to reduction and alkylation using dithiothreitol and iodoacetamide before LysC and trypsin digestion. Peptide mixtures were purified and desalted using C_{18}-stage tips. Peptide separation and sequence determination was performed with a nano-high

performance liquid chromatography system (Agilent 1100 series, Amstelveen, the Netherlands) connected to a 7-T linear quadrupole ion trap-ion cyclotron resonance Fourier transform mass spectrometer (Thermo Electron, Breda, the Netherlands). Peptides were separated on a 15-cm 100-µm-inner-diameter PicoTip emitter for online electrospray (New Objective, Woburn, MA) packed with 3 µm C18 beads (Reprosil, Dr Maisch GmbH, Ammerbuch-Entringen, Germany) with a 60-minute linear gradient from 2.4 to 40 percent acetonitrile in 0.5% acetic acid at a 300 nl/min flow rate. The four most abundant ions were sequentially isolated and fragmented in the linear ion trap by applying collisionally induced dissociation. Proteins were identified using the MASCOT search engine (Matrix science, London, UK) against the corresponding *S. pneumoniae* database. MSQuant was used for the quantification and determination of peptide ratios between wild-type and *psaR* mutant. A protein was considered differentially expressed when the results of 3 technical replicates showed at least a 1.5-fold change in protein abundance and a *P*-value <0.05 (one-sample *t*-test).

Infection models

Nine-week old female outbred CD-1 mice (Harlan, Horst, Netherlands) were used for all infection models. Prior to the infection experiments, D39 and TIGR4 (wild-type and *psaR*-mutants) were passaged in mice as described previously (31). Cultures of *S. pneumoniae* D39 and TIGR4 (wild-type and *ΔpsaR*) were grown in THY-broth to an OD_{600} of 0.3, and stored in aliquots in 10% glycerol at -80˚C. Prior to infection, these aliquots were spun down and bacteria were resuspended in sterile PBS to 10^6 CFU (colony forming units) in volumes depending on the infection model used. Upon intranasal infection, mice were anesthetized with 2.5% (vol/vol) isoflurane / O_2. At predetermined time points after infection, groups of mice were sacrificed by cervical dislocation and samples of various sites were taken to determine the bacterial load. During infection, signs of disease were closely monitored. If animals reached a moribund state, they were sacrificed by cervical dislocation and excluded from the experiment prematurely. All animal experiments were performed with approval from the Animal Experimentation Committee (DEC) of Erasmus Medical Centre, Rotterdam, The Netherlands.

Colonization model of infection

In the colonization model, 10 µl of PBS containing 10^6 CFU of bacteria was administered to the nostrils of groups of five mice as described previously (23, 30). Due to

this small volume, only the nose of the mice becomes infected. Bacteria were recovered from the nasopharynx by flushing the nose with 2 ml sterile PBS (31), and lungs were removed from the body and homogenized in 2 ml of sterile PBS using a hand held homogenizer (polytron PT 1200, Kinematica AG). Viable bacteria from the nasal lavage fluid and homogenized lung samples were counted by plating serial 10-fold dilutions on Colombia blood agar plates. Time points for sampling were 30 min, 24h, 48h, 96h, and 192h post-infection. The 30-min time point is considered to be the start of the infection, and is therefore referred to as t=0. Bacteriology results are expressed as geometric mean ± standard errors of the mean (SEM). Comparison of bacterial loads in the time-course experiment was performed using a Student's *t* test with $P < 0.05$ considered statistically significant.

Pneumonia model of infection

In the pneumonia model, five mice per group were infected with 50 µl of PBS containing 10^6 CFU of pneumococci as described previously (23). Bacteria were recovered from the different sites as described above, with the addition of a blood sample obtained by cardiac puncture. Time points for sampling were 0, 12, 24, and 36h post-infection. Viable bacteria isolated from the nasal lavage fluid, homogenized lungs, and blood were quantified as described above. Bacteriology results are expressed as geometric mean ± SEM. Comparison of bacterial loads in the time course experiment was performed using a Student's *t* test with $P < 0.05$ considered statistically significant.

Bacteremia model of infection

In the bacteremia model, groups of ten mice were infected in a tail vein with 10^6 CFU resuspended in 100 µl of sterile PBS as described previously (23). Bacteria were recovered from the blood by a lateral tail vein puncture from the same mouse at three predetermined time points after infection (0, 12, 24h) and by a cardiac puncture at the last time point, 36h. In addition, mouse survival times were scored, after which analysis of survival times was performed using the log-rank test with $P < 0.05$ considered statistically significant.

Accession numbers

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE13505.

Results

Transcriptional analyses of *ΔpsaR* in D39 and TIGR4

By means of microarrays analysis, we assessed which genes were affected in expression due to the mutation of *psaR* in two genetic backgrounds, i.e., D39 and TIGR4. To this end, transcriptional profiles of wild-type strains were compared to their isogenic Δ*psaR* strains. These bacteria were grown in chemically defined medium (CDM) and harvested at mid-log growth phase. In all experiments, the *psaR* mutant strains grew like wild-type. The concentration of Mn^{2+} in CDM is 180 μM, which is sufficient for PsaR-regulation (see reference (31), where 50 μM was used). For comprehensibility, loci of D39 are referred to by the TIGR4 gene identifiers (in Table 3 both annotations are given). Comparison of transcriptional profiles of D39 and TIGR4 wild-type with their Δ*psaR* counterparts revealed 19 differentially expressed genes in TIGR4Δ*psaR*, and 37 in D39Δ*psaR*. Of these, five genes were upregulated in both TIGR4 and D39 *psaR*-mutants, while only *psaR* itself was downregulated in both strains (Fig. 1).

The genes that were differentially expressed in both serotypes were all upregulated in the *psaR*-mutant confirming the general role of PsaR as a transcriptional repressor (28, 32). Among those were the previously described targets, the *psa* operon (*sp1648-sp1650*), *pcpA* (*sp2136*), and *prtA* (*sp0614*)**.** In addition, *sp1637,* encoding a hypothetical protein of unknown function, was found to be upregulated in the *psaR* mutants of both strains (Table 3).

Four genes were found to be repressed by PsaR in D39 (i.e., upregulated in D39Δ*psaR*) only: *sp0303*, encoding 6-phospho-beta-glucosidase, *sp0306*, encoding a putative transcriptional regulator, and two genes of a putative operon encoding a cellulose-specific phosphotransferase system (PTS) (*sp0308* and *sp0310*).

Twenty-seven genes were downregulated in D39*ΔpsaR*. This set of genes contained *sp0112*, predicted to encode an amino acid substrate-binding protein, the transcriptional regulator *mutR* (*sp0141*), a putative bacteriocin system (*sp0142-sp0146*), the *blp* two-component system (TCS13; *sp0526-sp0527*), the gene encoding response regulator CiaR (*sp0798*), and *glyA* (*sp1024*) encoding serine hydroxymethyltransferase. The full set of differentially expressed genes in D39 is listed in Table 3.

In addition to the common gene targets, nine genes were specifically upregulated in the *psaR*-mutant in TIGR4 (Fig. 1). These included the *rlrA* pathogenicity islet (*sp0461-sp0468*) as reported previously (28), *sp1636* (encoding an Rf2 family protein), and two

adjacent genes, encoding a MerR family transcriptional regulator (*sp1856*) and *czcD* (*sp1857*), a Zn^{2+}-efflux pump (Table 3).

Four genes were downregulated: *guaC*, encoding GMP reductase (*sp1249*), *ply*, encoding pneumolysin (*sp1923*), and two genes encoding the B and C component of a putative cellobiose-specific PTS (*sp2022* and *sp2023*). The complete set of differentially expressed genes in TIGR4Δ*psaR* is given in Table 3.

Proteome analysis of *ΔpsaR* in D39 and TIGR4

To examine if the observed PsaR-mediated differences in gene expression corresponded with changes in protein expression, we performed SILAC for both wild-type strains and their *psaR* mutants. To this end, wild-type and isogenic Δ*psaR* counterparts were cultured in the presence of stable isotope-labeled (heavy, $^{13}C_6$) or normal (light, $^{12}C_6$) L-lysine and L-arginine, respectively. Equal amounts of bacteria were mixed and analyzed by mass spectrometry, after which changes in protein expression between wild-type and isogenic Δ*psaR* strains were derived from the corresponding heavy to light peptide ratios.

In D39Δ*psaR*, nine proteins were found to be more abundant than in the wild-type (Table 3). Most pronounced were FtsY, PsaA, SufS, Cps2G, CysK, and PrtA, all of which displayed expression levels at least eight times higher in the *psaR*-mutant than in the wild-type. No proteins were identified that were less abundant in D39Δ*psaR* (Table 3).

In TIGR4Δ*psaR,* six proteins were more abundant than in the wild-type. Most pronounced were PcpA, PsaA, and RrgB, all with at least a four-fold increase in expression in the *psaR*-mutant (Table 3). Three proteins were less abundant in TIGR4Δ*psaR*, i.e., an ABC-transporter of a putative bacteriocin system (*sp0148*), TrxB, and ZmpB (Table 3).

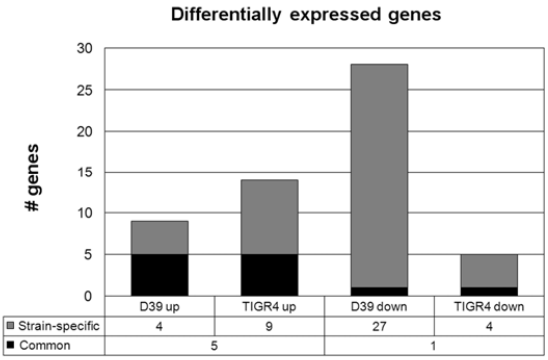

Figure 1. The number of genes differentially expressed in D39Δ*psaR* and TIGR4Δ*psaR*.

Table 3. Differentially expressed genes/proteins in *ΔpsaR* of D39 and/or TIGR4. Ratios are given as log_2-transformed expression of Δ*psaR* / wild-type.

Identifier		Gene	Annotation (www.kegg.com)	Microarray		SILAC	
TIGR4	D39			TIGR4	D39	TIGR4	D39
Upregulated							
sp0232	*spd0215*	*infA*	translation initiation factor IF-1				2.4
sp0303	*spd0277*	*bglA*	6-phospho-beta-glucosidase		1.1		
sp0306	*spd0280*		transcriptional regulator, putative		1.2		
sp0308	*spd0281*		PTS system, cellobiose-specific IIA component		1.5		
sp0310	*spd0283*		PTS system, cellobiose-specific IIC component		1.2		
-	*spd0322*	*cps2G*	glycosyl transferase, group 1 family protein				4.5
sp0459	*spd0420*	*pflB*	formate acetyltransferase			0.8	
sp0461	-	*rlrA*	transcriptional regulator	2.7			
sp0462	-	*rrgA*	cell wall surface anchor family protein	3.1			
sp0463	-	*rrgB*	cell wall surface anchor family protein	3.7		2.0	
sp0464	-	*rrgC*	cell wall surface anchor family protein	3.7			
sp0467	-		sortase SrtC, putative	2.5			
sp0468	-		sortase SrtD, putative	2.6			
sp0641	*spd0558*	*prtA*	serine protease, subtilase family PrtA	3.6	3.0		3.9
sp0869	*spd0764*	*sufS*	cysteine desulfurases, SufS subfamily protein				5.0
sp1244	*spd1101*	*ftsY*	signal recognition particle-docking protein FtsY				7.6
sp1517	*spd1345*	*greA*	transcription elongation factor GreA				2.9
sp1531	*spd1360*		hypothetical protein				2.7
sp1636	*spd1448*		Rrf2 family protein	1.3			
sp1637	*spd1449*		hypothetical protein	1.4	1.2		
sp1647	*spd1460*	*pepO*	endopeptidase O			0.6	
sp1648	*spd1461*	*psaB*	manganese ABC transporter, ATP-binding protein	3.6	3.3		
sp1649	*spd1462*	*psaC*	manganese ABC transporter permease	2.8	2.4		
sp1650	*spd1463*	*psaA*	ABC transporter, substrate binding lipoprotein			5.4	5.1
sp1805	*spd1591*		hypothetical protein			0.6	
sp1856	*spd1637*		transcriptional regulator, MerR family	1.2			
sp1857	*spd1638*	*czcD*	cation efflux system protein	1.7			
sp2136	*spd1965*	*pcpA*	choline binding protein PcpA	4.0	4.6	7.4	
sp2210	*spd2037*	*cysK*	cysteine synthase A				4.0
Downregulated							
sp0112	*spd0109*		polar amino acid transport system substrate-binding protein		-1.2		
sp0138	*spd0141*		hypothetical protein		-1.7		
sp0139	*spd0142*		hypothetical protein		-1.8		
sp0140	*spd0143*	*ugd*	UDP-glucose/GDP-mannose dehydrogenase		-1.7		
sp0141	*spd0144*	*mutR*	positive transcriptional regulator of MutA		-1.2		
sp0142	*spd0145*		hypothetical protein		-1.0		
sp0143	*spd0146*		hypothetical protein		-1.9		
sp0144	*spd0147*		hypothetical protein		-1.5		
sp0145	*spd0148*		hypothetical protein		-2.0		
sp0146	*spd0149*		hypothetical protein		-1.7		
sp0148	*spd0150*		ABC transporter, substrate-binding protein			-2.1	
sp0524	*spd0466*		BlpT protein, fusion		-1.5		
sp0525	*spd0467*	*blpS*	BlpS protein		-1.4		
sp0526	*spd0468*	*blpR*	response regulator BlpR (TCS13)		-1.2		
sp0527	*spd0469*	*blpH*	sensor histidine kinase BlpH, putative (TCS13)		-1.1		
sp0529	*spd0471*	*blpB*	BlpC ABC transporter		-1.5		
sp0530	*spd0472*	*blpA*	BlpA, pseudogene		-1.5		
sp0533	*spd0046*	*blpK*	bacteriocin BlpU (highly similar to *sp0533*)		-1.5		
sp0541	*spd0046*	*blpO*	bacteriocin BlpO (5' highly similar to *sp0541*)		-1.5		
sp0545	*spd0473*	*blpY*	immunity protein BlpY		-2.4		
sp0546	*spd0474*	*blpZ*	BlpZ protein, fusion		-1.2		
sp0547	*spd0475*		hypothetical protein		-2.4		
sp0664	*spd0577*	*zmpB*	zinc metalloprotease ZmpB, putative			-0.8	

sp0798	*spd0701*	*ciaR*	DNA-binding response regulator CiaR		-1.1	
sp0925	*spd0817*		hypothetical protein		-1.4	
sp1024	*spd0910*	*glyA*	serine hydroxymethyltransferase		-1.1	
sp1249	*spd1107*		GMP reductase	-1.0		
sp1458	*spd1287*	*trxB*	thioredoxin reductase			-1.0
sp1543	*spd1372*		glyoxalase family protein		-1.5	
sp1638	*spd1450*	*psaR*	iron-dependent transcriptional regulator, PsaR	-2.7	-2.0	
sp1802	*spd1588*		hypothetical protein		-1.2	
sp1804	*spd1590*		general stress protein 24, putative		-1.3	
sp1923	*spd1726*	*ply*	pneumolysin	-1.0		
sp2022	*spd1831*		PTS system, cellobiose-specific IIC component, putative	-1.4		
sp2023	*spd1832*		PTS system, cellobiose-specific IIB component, putative	-1.2		

PsaR does not contribute to pneumococcal colonization

To assess the contribution of PsaR-regulation to pneumococcal virulence, we examined the phenotypes of the two wild-type strains and their *psaR* mutants in three murine models of infection.

In the colonization model, both wild-type D39 and TIGR4 were capable of extended colonization of the murine nasopharynx for a period of 192h. The level of colonization was fairly consistent during this period, varying between 10^4 - 10^6 CFU/ml (Fig. 2).

We did not observe a clear effect of the deletion of *psaR* on the colonization ability of strains: comparable bacterial loads of wild-type and mutant strains were found during 192h of colonization, with bacterial loads between 2.5 x 10^4 CFU/ml to 3.2 x 10^5 CFU/ml for D39 wild-type and its isogenic *psaR*-mutant, and 1.3 x 10^5 CFU/ml to 5.0 x 10^5 CFU/ml for TIGR4 wild-type and its *ΔpsaR* derivative (Fig. 2A and B). The only exception was a small but significantly higher load (P=0.0049) of D39Δ*psaR* at 24h (Fig.2B).

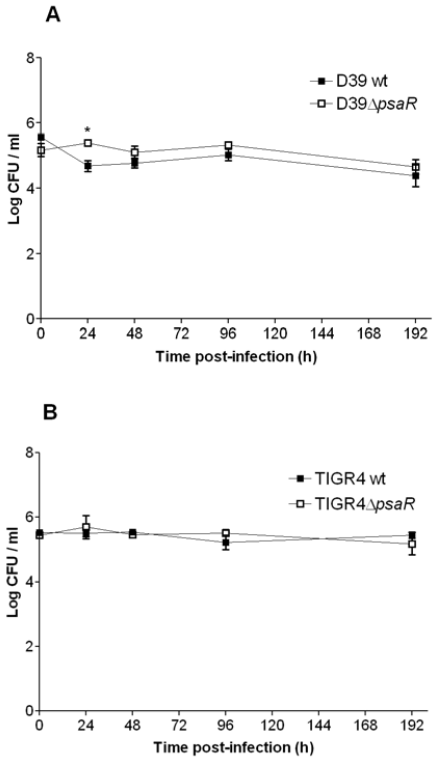

Figure 2. Colonization model. Bacterial loads in the nasal lavage fluid of mice infected with **(A)** D39 wild-type and D39Δ*psaR*, **(B)** TIGR4 wild-type and TIGR4Δ*psaR*. * indicates $P < 0.05$.

PsaR is not required for pneumococcal pneumonia

In the pneumonia model, we observed a similar trend in bacterial loads of the nasopharynx as was observed in the colonization model, although the actual numbers of bacteria were lower. At 12h of infection there was a small but significantly higher nasopharyngeal load in mice infected with D39Δ*psaR* as compared to wild-type (data not shown). For TIGR4 wild-type and Δ*psaR*, small but significant differences in bacterial load were observed throughout the entire experiment (data not shown). Furthermore, bacterial loads in the nasopharynx were comparable to those of the first 36h in the colonization model (data not shown).

We did not observe any clear difference in the bacterial survival in the lungs between wild-type and *psaR* mutants (Fig. 3A). However, immediately after infection (0h) and 12h post-infection, the lung homogenates of D39Δ*psaR*-infected mice had significantly higher bacterial loads than those of wild-type infected mice: 1.2×10^5 vs 6.3×10^4 CFU/ml at 0h, and

1.5 x 10^5 vs 5.8 x 10^4CFU/ml at 12h. This suggests that there might be an initial positive effect of the *psaR*-mutation, as the inoculum of the wild-type contained (more than) twice as many bacteria than that of the *psaR*-mutant (1.2 x 10^6 and 4.5 x10^5 and CFU/ml, respectively).

In contrast, mice infected with the TIGR4 *psaR*-mutant had lower bacterial loads in the lungs compared to mice infected with TIGR4 wild-type, although these differences were only statistically significant at 24h post-infection (P=0.0401) (Fig. 3B).

The number of bacteria that were able to reach the systemic circulation was not significantly different between wild-type and *ΔpsaR* in either D39 or TIGR4 (data not shown), indicating that PsaR-regulation is not required for the transition from the lungs to the systemic circulation.

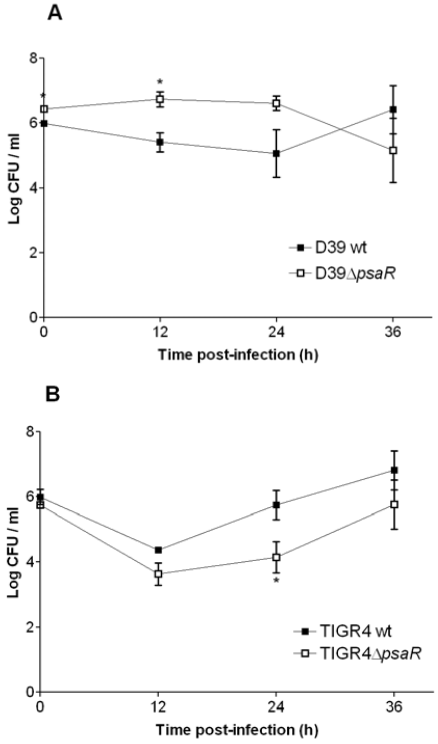

Figure 3. Pneumonia model. Bacterial loads in lungs and blood of mice infected with **(A)** D39 wild-type and D39Δ*psaR*, **(B)** TIGR4 wild-type and TIGR4Δ*psaR*. * indicates $P < 0.05$.

PsaR contributes to survival of pneumococci during bacteremia

The most prominent phenotype of the *psaR*-mutants was observed in the bacteremia model of infection. D39 wild-type-infected mice had significantly more bacteria in the blood

than the D39Δ*psaR*-infected mice during the first 24h of infection (Fig. 4A), while variation in the inocula was marginal (1.6 x 10^6 for wild-type and 1.4 x 10^6 for *psaR*-mutant). In contrast, no differences were seen between mice infected with TIGR4 wild-type and TIGR4Δ*psaR* during the first 24h of infection. At 36h post-infection, however, TIGR4 wild-type-infected mice had twice as many bacteria in the bloodstream (*P*=0.0396) (Fig. 4B).

In addition to the differences in bacterial load, we also observed a difference in murine survival after infection with D39 or TIGR4 in our bacteremia model: All TIGR4-infected mice (wild-type and Δ*psaR*) survived, in contrast with D39 wild-type infected mice, which died within 30h. Of the D39Δ*psaR*-infected mice, three survived the experiment. Consequently, the median survival time of D39Δ*psaR*-infected mice was significantly longer than that of mice infected with its wild-type parental strain (the median survival for Δ*psaR* was 24h and for wild-type 30h, with *P*=0.0405 and hazard ratio=1.866).

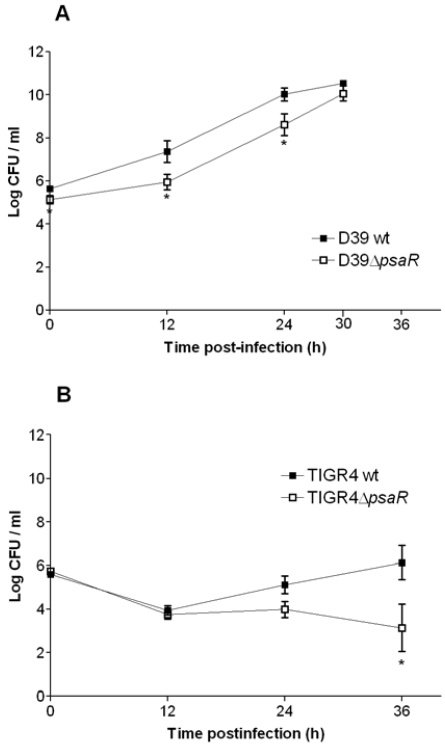

Figure 4. Bacteremia model. Bacterial loads in blood of mice infected with **(A)** D39 wild-type and D39Δ*psaR*, **(B)** TIGR4 wild-type and TIGR4Δ*psaR*. * indicates $P < 0.05$.

Discussion

During colonization and infection of the human host, *S. pneumoniae* encounters fluctuating amounts of free Mn^{2+} at the different niches where the bacterium resides. Since Mn^{2+} is required for several cellular processes, proper regulation of manganese homeostasis is vital for pneumococcal physiology and virulence. The transcriptional repressor PsaR has been described to play an important role in this process, at least in the genetic background of strain EF3030 (serotype 19F) (28). However, we and others have previously shown that, even though transcriptional regulators themselves appear to be conserved between *S. pneumoniae* strains, they often have strain-specific impact on global transcription and virulence (4, 24, 40). To examine whether a similar strain-specificity holds true for PsaR, we identified its targets and its contribution to experimental virulence in two additional strains, TIGR4 and D39.

We used a combination of transcriptional and proteome analyses for the identification of PsaR targets. Observed discrepancies between these two approaches could be the result of several factors, such as low levels of gene expression, protein instability, or post-translational regulation. The previously reported PsaR targets, the Psa operon, *pcpA*, and *prtA* (28, 32), were confirmed in TIGR4 and D39 by both transcriptomics and proteomics. In addition, we found *sp1637*, encoding a hypothetical protein, to be upregulated in both *psaR* mutants. Since the latter gene is located directly upstream of *psaR* (*sp1638*), we cannot entirely exclude the possibility that the derepression of *sp1637* is caused by transcriptional read-through of the trimethoprim resistance cassette used to create the *psaR* knockout. We have previously shown that the common PsaR target *pcpA* is required for adherence to the human Detroit epithelial cell line and that the expression of this gene is also directly positively regulated by the nutritional regulator CodY (22). The inability of the *codY*-mutant to colonize the murine nasopharynx underscored, albeit indirectly, the involvement of PcpA in adherence and colonization. However, a recent study showed that PcpA is not involved in colonization but has a role in invasive disease (15). These contradictory results are possibly due to different experimental set-ups, however, the exact role of PcpA during pneumococcal pathogenesis remains unclear.

In addition to the shared PsaR-targets (i.e., the overlapping genes between D39 and TIGR4), several genes were differentially expressed in D39 or TIGR4 only. This strain-specific differential expression might be due to direct regulation by PsaR, but is more likely to be either an indirect effect caused by an imbalance in Mn^{2+}/$Zn2^{+}$-homeostasis due to the lack of PsaR or, possibly, downstream signaling of other regulators. Moreover, it seems that this

effect was more severe for D39, since more genes and proteins were affected in their expression level in this strain. Downstream regulation or a disturbed balance in other cellular processes might be the cause of this differential expression, since manganese cations have been shown to be required for several bacterial cellular processes (26). For example, manganese cations have been shown to function as co-factors for enzymes in glycolysis, amongst others 6-phospho-β-glucosidase (50). The genes encoding 6-phospho-β-glucosidase and a phosphotransferase system (PTS) downstream of it (*sp0303*, *sp0306*, and *sp0308*), were upregulated in D39*ΔpsaR*, in line with indirect regulation. However, in D39 these genes are strongly downregulated in the presence of Zn^{2+}, which is not in agreement with the opposite effect of Mn^{2+} and Zn^{2+} on PsaR-regulation (32).

In D39*ΔpsaR*, the gene *ciaR*, involved in competence development, was upregulated. It has been reported that Mn^{2+} is required for genetic transformation (12), suggesting that downregulation of this gene might be a indirect effect of the *psaR*-mutation. Several other systems were downregulated in D39*ΔpsaR* alone, such as a putative bacteriocin system (*sp0142-sp0146*), the *blp* bacteriocin system, and the *blp* two component system (TCS13). The genes *sp0141-sp0146* have also been shown to be regulated by the nutritional regulator CodY in D39 (22). Possibly, the downregulation of the *blp* bacteriocin genes is due to downregulation of the *blp* two component system (10, 11).

Notably in TIGR4, a MerR family regulator (*sp1856*) and *czcD* (*sp1857*), encoding a Zn^{2+}-efflux system, were upregulated in the *psaR*-mutant. These two genes have also been shown to be upregulated in the presence of Zn^{2+}, underscoring the reported opposite effect of Mn^{2+} and Zn^{2+} on PsaR-regulation (32). The *czcD* gene has recently been shown to be regulated by SczA (*sp1858*) in reaction to increasing cellular zinc concentrations (34), but this efflux system might also be involved in Mn^{2+}-homeostasis. This again indicates that these regulatory systems of cation homeostasis are intertwined (32).

The expression of *ply* (encoding the pneumococcal toxin pneumolysin) was downregulated as a result of the *psaR* mutation during *in vitro* growth in TIGR4 only. If this downregulation also occurred during our infection experiments, it did not have a large effect on experimental virulence of the TIGR4 strain, as the Δ*psaR* mutant was as virulent as the wild-type in all our infection models. Only at 24h post-infection during experimental pneumonia, mice infected with the *psaR*-mutant had significantly lower bacterial loads in the lungs compared to those infected with wild-type.

A role for PsaR in virulence has been reported in an EF3030 genetic background (28). During seven days of colonization no difference between the wild-type and isogenic *psaR*-

mutant was observed, which is in agreement with our results. Johnston *et al.* (2006) reported that during lung infection the *psaR*-mutant had significantly lower bacterial loads than the wild-type after 7 days of infection. However, in our pneumonia model, we observed higher bacterial loads in D39*ΔpsaR* -infected mice at the beginning of the experiment (the first 12h). The TIGR4 *ΔpsaR*–infected mice had significantly lower bacterial loads at 24h only. Taken together, we did not observe any clear role for PsaR during lung infection in our study.

Upon intravenous infection, we observed the most pronounced effect of deletion of *psaR*. The *psaR*-mutant was attenuated at the early stages of blood infection. In line with this, PsaR was identified by a large STM study to be required for full virulence of a serotype 3 strain in a bacteremia model of infection after 24h of infection (36). Importantly, for D39 no significant difference was observed in our model at 30h post-infection. This indicates that in the first 24h PsaR-regulation is required for adaptation to the blood. Furthermore, in D39, survival times of wild-type and Δ*psaR*-infected mice were different, although this just reached statistical significance. In contrast, mice infected with TIGR4 wild-type and *ΔpsaR* did not exhibit any differences in the first 24h of experimental bacteremia, while after 36h they did. This indicates that in TIGR4, PsaR contributes to the later stages of bacteremia. However, since the TIGR4 wild-type was not able to cause infection as severe as D39, this contribution is considered to be marginal.

Although we observed disturbed gene regulation in the D39 and TIGR4 mutants, in most infections models (described above) we did not observe large effects on virulence. A study performed on the pneumococcal carbohydrate regulator RegM showed that, even though deletion of a regulator does not necessarily lead to large differences in expression of known virulence genes, it can affect pneumococcal virulence (14). Conversely, a second study by Trombe and co-workers showed that deleting the global regulator RegR, does not dramatically affect virulence, but has a large effect on gene expression (7). It seems that the pneumococcus has several compensatory virulence pathways.

Strain-specific contributions of genes encoding transcriptional regulators have been reported previously and complicate ascribing a general role for these genes in transcriptional control of their targets and their contribution to virulence (24, 40). PsaR directly controls the expression of a select group of genes individually important for virulence (*psaBCA* and *rlrA*) (28, 32). The observed differences in PsaR-regulated gene expression between D39 and TIGR4 might be the indirect effect of an unbalanced Mn^{2+}-homeostasis, which suggests that physiological differences might reflect genetic differences present in these strains. A clear example of the genetic difference is the *rlrA* pathogenicity islet, which is strongly upregulated

in TIGR4Δ*psaR*. However, the genetic diversity between strains only varies as much as 10%, most of which can be attributed to the capsular genes (16, 35). In conclusion, PsaR does not contribute to colonization of *S. pneumoniae*, but it is involved in invasive disease where it has a strain-specific impact during both pneumonia and bacteremia.

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CHAPTER 4

CodY of *Streptococcus pneumoniae*: link between nutritional gene regulation and colonization

The CodY regulon of *Streptococcus pneumoniae*

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Abstract

CodY is a nutritional regulator mainly involved in amino acid metabolism. It has been extensively studied in *Bacillis subtilis* and *Lactococcus lactis*. We investigated the role of CodY in gene regulation and virulence of the human pathogen *Streptococcus pneumoniae*. We constructed a *codY*-mutant and examined the effect on gene and protein expression by microarray and 2D DIGE analysis. The pneumococcal CodY-regulon was found to consist predominantly of genes involved in amino acid metabolism, but also several other cellular processes, such as carbon metabolism and iron uptake. By means of electrophoretic mobility shift assays and DNA footprinting, we showed that most targets identified are under direct control of CodY. By mutating DNA predicted to represent the CodY-box based on the *L. lactis* consensus, we demonstrated that this sequence is indeed required for in vitro DNA-binding to target promoters. Similar to *L. lactis*, DNA-binding of CodY was enhanced in the presence of branched chain amino acids, but not by GTP. We observed in experimental mouse models that *codY* is transcribed in the murine nasopharynx and lungs, and is specifically required for colonization. This finding was underscored by the diminished ability of the *codY*-mutant to adhere to nasopharyngeal cells in vitro. Furthermore, we found that *pcpA*, activated by CodY, is required for adherence to nasopharyngeal cells, suggesting a direct link between nutritional regulation and adherence. In conclusion, pneumococcal CodY predominantly regulates genes involved in amino acid metabolism and contributes to the early stages of infection, i.e. colonization of the nasopharynx.

Introduction

Bacteria encounter various environmental cues during their life cycle to which they need to respond appropriately in order to survive. Different niches within a host are likely to provide different nutritional challenges to the microorganism. Such alterations in bacterial gene expression in response to environmental changes are under strict control of transcriptional regulators.

CodY is a global nutritional repressor, which is highly conserved in low G+C bacteria and has been investigated extensively in *Bacillus subtilis*, *Lactococcus lactis*, *Streptococcus pyogenes* and very recently in *Listeria monocyogenes* (3, 16, 33, 35, 39, 42). *B. subtilis* CodY represses the transcription of over a 100 genes during exponential growth, which are involved in different metabolic pathways and cellular processes, such as peptide uptake, development of genetic competence, branched-chain amino acid biosynthesis, motility, and sugar uptake (35, 38). There is one exception where CodY functions as an activator in *B. subtilis*: the gene encoding acetate kinase is positively regulated by CodY and the carbon regulator CcpA, possibly ensuring that an overflow pathway for carbon metabolism is active (40).

Functional studies have shown that the DNA-binding activity of *B. subtilis* CodY is enhanced by both GTP and the branched-chain amino acids (BCAAs) isoleucine, leucine, and valine (23, 41). The crystal structure of two fragments of *B. subtilis* CodY, containing its cofactor and DNA binding domains, revealed that the regulatory protein interacts with DNA as a dimer (31). *L. lactis* CodY DNA-binding to its recently identified binding consensus is enhanced by BCAAs, but not by GTP (13, 14, 17, 36). Differences between these needs for cofactors of *B. subtilis* and *L. lactis* CodY might reflect the physiology of these bacteria. For instance, GTP plays a major role in the development of sporulation in *B. subtilis*, a process absent from the life cycle of *L. lactis*. Moreover, in *B. subtilis*, low levels of GTP induce development of competence by relieve of CodY-repression of *comK,* a critical competence regulator (20, 38). In contrast, natural transformation has never been observed in *L. lactis*. Interestingly, the lactococcal genome sequence revealed the presence of orthologues of several genes involved in natural transformation in other bacteria (6, 29), but none of these putative competence genes belong to the lactococcal CodY-regulon (14).

Another process in which GTP plays a major part is the stringent response. This is a response of a bacterium to amino acid starvation, during which the signal molecule ppGpp is accumulated, resulting in a shut down of the synthesis of many rRNAs and tRNAs (21, 42). An essential factor in the accumulation of ppGpp is the ribosome-bound protein RelA, which converts GTP to ppGpp. In *S. pyogenes* a RelA-independent response to amino acid starvation

is found that is, at least in part, regulated by CodY (44, 45). Among the genes repressed during this response are virulence factors such as those encoded by *graB*, *speB*, *speH* (33). Interestingly, CodY induced expression of *pel/sagA* and *mga*, genes encoding regulatory proteins that themselves positively affect the expression of numerous other virulence factors. This observation suggests a clear link between nutritional regulation and virulence in *S. pyogenes* (33).

Streptococcus pneumoniae (the pneumococcus) is a human pathogen, which causes diseases such as meningitis, pneumonia, and otitis media, in the young, elderly and immuno-compromised (5). Pneumococcal disease is preceded by colonization of the nasopharynx, which is asymptomatic. From there it can develop into disease under the appropriate conditions. Analysis of the genomes of *S. pneumoniae* R6 (19), D39 (30), and TIGR4 (46) revealed that CodY orthologs are present on the chromosomes of these strains (*spr1439* for R6, *spd1412* for D39, and *sp1584* for TIGR4). Here, we report on the physiological role of CodY in *S. pneumoniae* D39 in global transcription, translation, and DNA-binding. We show that the pneumococcal CodY-regulon consists mainly of genes that are involved in amino acid metabolism, biosynthesis, and uptake. Binding of CodY to its target promoters requires a 15-bp recognition site, and is enhanced by BCAAs but not by GTP. Furthermore, we demonstrate that CodY is required for optimal levels of *in vitro* adherence and colonization of the murine nasopharynx.

Materials and Methods

Bacterial strains and media

Bacterial strains and plasmids used in this study are listed in Table 1. All pneumococcal strains used in this study were grown in Todd-Hewitt Yeast broth at 37˚C or on Colombia base agar supplemented with 5% sheep blood (Biotrading). Pneumococcal strains were maintained in 10% glycerol, 10% skim milk at -80˚C. *Escherichia coli* DH5α (Table 1) was grown in Luria Broth at 37˚C while shaking or on Luria Broth agar supplemented with appropriate antibiotics (50 mg/l ampicilin and/or 20 mg/l trimethoprim).

Table 1. Bacterial strains and plasmids used in this study.

Strains	Antibiotic resistance	Reference
E. coli		
BL21 (DE3)		Stratagene
DH5α		Stratagene
S. pneumoniae		
D39 wild-type		NCTC 7466
D39 Δ*codY*	trimethoprim	this study
D39 Δ*cps*	kanamycin	(7)
D39 Δ*cps*Δ*codY*	kanamycin; trimethoprim	this study
D39 Δ*cps*Δ*pcpA*	spectinomycin; kanamycin	this study
D39 Δ*cps*Δ*codY*Δ*pcpA*	kanamycin; trimethoprim; erythromycin	this study
Plasmids		
pBluescript KS+		Stratagene
pCR2.1		Invitrogen
pET11C		New England Biolabs
pR412T7		(4)
pKOT		This study
pKOCOD		This study

Construction of mutant strains

The gene encoding *codY* (*spd1412*) was deleted from strain D39 by allelic replacement with the *dfr13* cassette conferring trimethoprim resistance (2). To this end, *codY* with 1000 bp of upstream and downstream flanking sequences was amplified from chromosomal D39 DNA using primer pair CodSacFwd and CodKpnRv (Table 2). This amplicon was cloned into pBlueScript KS+. Coding DNA of *codY* was deleted from the plasmid by performing an inverse PCR with primer pair CodHindFwdinv and CodPstRvinv, amplifying the *codY*-flanking sequences and pBlueScript KS+ and introducing HindIII and PstI restriction sites for further cloning. This amplicon was ligated with the *dfr13*-cassette excised from pKOT with

HindIII and PstI to create the knockout construct pKOCOD, and transformed to *E. coli* DH5α. A 2660-bp linear DNA fragment containing *codY*-flanking DNA and *dfr13* was amplified from pKOCOD using primer pair CodSacFwd and CodKpnRv. This PCR product was used to delete *codY* from the genome of *S. pneumoniae* D39 by CSP-1-induced (100 ng/ml) transformation. Transformants were selected on the basis of trimethoprim-resistance and were checked by PCR for recombination at the desired location on the chromosome. Wild-type D39 was subsequently transformed with chromosomal DNA isolated from these transformants to rule out the possibility of any additional mutations on the chromosome.

The *pcpA* (*spd1965*) deletion mutants were constructed by allelic replacement with the spectinomycin-resistance cassette of plasmid pR412T7 as follows. Primers pcpA_L1/pcpA_L2 and pcpA_R1/pcpA_R2 were used to generate PCR products of the left and right flanking regions of *pcpA* (approximately 500 bp each) (Table 2). These PCR products were fused to the spectinomycin-resistance gene amplified with primers pR412_L and pR412_R by means of overlap extension PCR. The resulting PCR product was transformed to *S. pneumoniae* D39Δ*cps* and transformants were checked for the presence of the mutation by PCR.

Transcriptional profiling of D39Δ*codY*

Microarray analysis was performed essentially as described (18). In short, 500 ml of THY-broth was inoculated with 10-20 colonies from agar plates, and these cultures were statically grown at 37°C. In all experiments, D39 wild-type and Δ*codY* displayed comparable growth characteristics. Samples for RNA isolation were taken when the cultures reached an optical density (OD_{600}) of either 0.1 or 0.2 (early and mid-log growth, respectively). RNA was isolated and purified using the High Pure RNA isolation kit (Roche diagnostics) as described (18). Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Roche diagnostics). RNA was isolated from three replicate cultures. Synthesis, subsequent labeling of cDNA, and microarray hybridization was performed as described (18, 48). In all cases, dye-swapping was performed with one of the three biological replicates. Microarrays used in this study were constructed as described (18, 28) and contain PCR amplicons representing 2,087 ORFs of *S. pneumoniae* TIGR4 and 184 ORFs unique for *S. pneumoniae* R6, all spotted in duplicate.

Table 2. Oligonucleotide primers used in this study.

Name primer	Nucleotide sequence 5' - 3' (restriction sites are underlined)	Restriction site
Cloning		
CodSacFwd	GCGCGCCCGCGGAGGTCGTGCTGGTAAGTCAG	*Sac*II
CodKpnRv	GCGCGCGGTACCGATGATTTTCAGGCCAGATG	*Kpn*I
CodHindFwdinv	TACGAAGCTTTGAGTCTGCGGGGATTATTG	*Hind*III
CodPstRvinv	ATGCCTGCAGGTGCCATTTTTCACCTCGAA	*Pst*I
CodKOrv	CATATTAGCCCCTTGAACGTAGTC	-
Cod 5' chrom	CATGTCAAGATTGCGGCTAA	-
pcpA_L1	GTTTCCCAGAAAGACTCTGG	
pcpA_L2[a]	**CCACTAGTTCTAGAGCGGC**GGAGTATCTGCTAGGATTGG	
pcpA_R1	ATAAGAATACGGATTCGGACG	
pcpA_R2[a]	**GCGTCAATTCGAGGGGTATC**GATGGCTATCGAGTCAATGC	
pR412_L	GCCGCTCTAGAACTAGTGG	
pR412_R	GATACCCCTCGAATTGACGC	
Overexpression		
CodY-NheI-H6-Fw[b]	CGGCTAGC**CATCACCATCACCATCAC**GCACATTTATTAGAAAAAACT	*Nhe*I
CodYBamRv	CGCGCGGGATCCTTAGTAATCTCTTTTCTT	*Bam*HI
EMSA		
codY EMSA fw	TGCAAGTCAATAAGGAATTTTCA	-
codY EMSA rv	GCCATTTTTCACCTCGAATT	-
gdhA EMSA fw	CCAAAAACTGAATTGAAAGAATTT	-
gdhA EMSA rv	CTTTAGCAGATGTCATATCGTTCTCC	-
amiA EMSA fw	GACACTTCGAACGACAATTTG	-
amiA EMSA rv	TGACAACCATTATATCACATTATCCA	-
ilvB EMSA fw	CATAAATAAACGTTAAAATAGAAAATTCAG	-
ilvB EMSA rv	CCCTTTCTTTCCTCTTAAAAATAAC	-
ilvE EMSA fw	GAAATGAAGAATCAGTTCTAAGATGG	-
ilvE EMSA rv	TCCCAATCAATCGTTACTGTCA	-
livJ EMSA fw	CCCTTTGTGGGCAATCTTTA	-
livJ EMSA rv	CAAGCGCCACAAACGATA	-
psaR EMSA fw	TGAAAGAAGAGCTATTTTCGTCAT	-
psaR EMSA rv	CTTTGTTTGGGGTCATTCGT	-
livH EMSA fw	TCAACGTCGCCTTGGATTAT	-
livH EMSA rv	CGAGGGTTTTCCCTCACTTT	-
acuB EMSA fw	TTCAGAGCTCTTTTTGCTAGCTT	-
acuB EMSA rv	CCTTGCGGGTCATAAAATCT	-
asd EMSA fw	CCCTAGTCTAGCGACTGGGATT	-
asd EMSA rv	GCGCCGACTACAGCAACT	-
gapN EMSA fw	CGCCTTGACGTAGTGGATTT	-
gapN EMSA rv	TCGGATGATTTCCATTTTCC	-
aliB EMSA fw	TTGAACAATCTTTTAGGAGAACTTGA	-
aliB EMSA rv	CATTTCCAGAACCTCCTGCT	-
fatD EMSA fw	TCCCTCGAAGATATTATTTTATCAGA	-
fatD EMSA rv	TTTCATACCCCGTCCTTTCA	-
spr0140 EMSA fw	GACATTCTATTTAGAACGAGGATTGA	-
spr0140 EMSA rv	ATTCCCCCAGTTCCATTTTT	-
spr0141 EMSA fw	TCTATCAAAATCGCAAATAAGAAA	-
spr0141 EMSA rv	TTCCATTGTTTCTGCAAATTGT	-
spr0157 EMSA fw	AACAATGTTTTAGAAGCAAAGGTG	-
spr0157 EMSA rv	GCTTGCGACATGATAAATACTCC	-
spr0788 EMSA fw	GAGGAAGGCCTTGTCCAGTT	-
spr0788 EMSA rv	CCCATAGAGCAACCTGTCGT	-

spr1436 EMSA fw	TGCGTAAACTACGTGAGCAA	-
spr1436 EMSA rv	TGACCTGCTTCTGACATTTGA	-
spr1934 EMSA fw	CTCCGGTTGCTTGTCTCAGT	-
spr1934 EMSA rv	AGCAGTCCCTCCACGTGATA	-
spr1945 EMSA fw	TGTGTTTATGGAGAGATGACAATTT	-
spr1945 EMSA rv	AACCGCAGCTGTAGTTAATGA	-
spr1982 EMSA fw	CTTGGTCAGGGTCAAGGAAG	-
spr1982 EMSA rv	TCGCCATAAGTGTGTTCCTG	-
fake gdhA EMSA fw2[c]	GAATTGAAAGGGTCTCGAGCTGCTATCTGTTTTTTC	-
psaR EMSA codY fw2[d]	GCTATTTTCAATTTTTAGAAAATTTCGTTTTTTC	-
Footprinting		
codY FP fw	GCAACTTGTCAATAGAAAAGGAA	-
fake gdhA FP fw	GATATTTCCAAGAAAAACGTTCG	-
RT PCR		
codYF	GATTGCCAGTACCGTTGT	-
codYR	CACGGAGTTCGGAGTAAG	-
gyrAF	TCTTGATTGCGCTAGACC	-
gyrAR	ACGACGAAGACGCATATC	-

[a] Overlap with the primers pR412_L and pR412_R in bold.

[b] 6xHis-tag is indicated in bold.

[c] Underlined sequence indicates random nucleotides used to replace the putative CodY-box of the *gdhA* promoter.

[d] Underlined sequence indicates the CodY-box introduced into the *psaR* promoter.

DNA microarray data analysis

Dual-channel array images were acquired with a GeneTac LS IV confocal laser scanner (Genomics Solutions) and analysed with ArrayPro 4.5 software (Media Cybernetics Inc.). Spots were screened visually to identify those of low quality. Slide data were processed using MicroPreP as described (15, 18, 49). Prior to analysis, automatically and manually flagged spots and spots with very low background subtracted signal intensity (5% of the weakest spots (sum of Cy3 and Cy5 net signals)), were filtered out of all datasets. Net signal intensities were calculated using a grid-based background subtraction. A grid-based Lowess transformation was performed for slide normalization, negative and empty values were removed, and outliers were removed by the deviation test. Further analysis was performed using a Cyber-T Student's *t* test for paired data (32). For identification of differentially expressed genes, only genes with a minimum of six reliable measurements, a Bayesian *p*-value < 0.001, a False Discovery Rate (FDR) < 0.05, and a standard deviation < ratio were included. Since these criteria are purely a statistical measure of differential gene expression and reproducibility across replicates, an additional fold-change cut-off of 2 was applied.

Initial analysis indicated that the set of genes regulated by CodY at OD_{600} 0.1 and 0.2 was similar, i.e., 33 genes were upregulated at both optical densities, 4 genes upregulated at 0.1 and not at 0.2, and 3 genes upregulated at 0.2 and not at 0.1. Because growth at these phases is also more or less identical (exponential growth), datasets for both optical densities were combined. Sequences of several differentially expressed genes were analyzed using TMHMM on the CBS Prediction Server for transmembrane domains (www.cbs.dtu.dk/services/TMHMM/).

2D DIGE

The three independent pneumococcal cultures used for transcriptional profiling (described above) were also used for proteome analysis. Pneumococcal cells were harvested by centrifugation at 4˚C and washed 4 times with cold PBS containing 1 mM PMSF. The pellet was resuspended in 500 µl of milliQ, and stored at -20˚C until further use. Sample preparation and Cy-labeling of proteins was performed according to the manufacturer's protocol (www.amershambiosciences.com). In short, 50 µg protein of both wild-type and mutant strains was labeled with Cy3 and Cy5, respectively. After labeling, an additional 200 µg protein of the corresponding strain was added to have sufficient material for spot identification by MALDI-TOF. Fluorescently labeled protein samples were combined, and the total of 500 µg protein was isoelectric focused on 18 cm Immobilized pH4-7 gradient strips (Amersham). For separation in the second dimension, 12-20% gradient polyacrylamide gels were used. Gels were scanned on a Typhoon 9410 imager (Amersham Biosciences) and analyzed using Z3 software (Compugen). Spots that showed at least a 2-fold change in protein abundance were selected and cut out of the gel after visualization by Coomassie staining. Tryptic digests of proteins were analysed by MALDI-TOF using the UltraFlex Mass-spectrometer (Bruker). Mascot Search software (Matrix Science) was used for identification of the proteins. Ratios were calculated from duplicate gels of the three biological replicates. Average ratios were only calculated from spots showing at least a 2-fold change in abundance in at least four out of six gels.

Overexpression of pneumococcal CodY in *E. coli* and purification of CodY

The gene *codY* was PCR-amplified using primer CodY-NheI-H6-Fw and CodYBamRv (Table 2) and cloned into pCR2.1. Using the restriction sites NheI and BamHI introduced on the PCR-product, the H_6-codY (H_6 = His-tag) was then cloned in the NheI/BamHI site of pET11C and transformed to *E. coli* BL21 (DE3) for overexpression. The

codY coding sequence was confirmed by sequencing. Purification of the N-terminal His_6-tagged CodY (H_6-CodY) was performed as previously described using the HisTrap Kit (Amersham Biosciences) by means of Ni-affinity chromatography (1). The purified protein was dialyzed against 60 mM NH_4HCO_3, freeze-dried and stored at -20˚C until further use. The identity of the purified protein was confirmed using MALDI-TOF analysis and the concentration was determined by the BCA-assay (Biorad).

Electrophoretic mobility shift assay

Gel mobility shift assays were performed essentially as previously described (13). Briefly, DNA-fragments of several upstream regions were PCR-amplified in the presence of [α-^{32}P]dATP (10 µCi, 3000 Ci/mmol per 50 µl reaction volume, MP Biomedicals) using primers shown in Table 2. Subsequently, 0.4 ng of radioactive amplicon was added to a 50-µl reaction mixture containing binding buffer (20 mM Tris-HCl (pH 8), 8.7% (vol/vol) glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 250 mM KCl, 0.5 mM DTT, 2 µg BSA), and purified CodY in concentrations of 0, 100, 250, 500, 1000, or 2000 nM. Branched-chain amino acids (leucine, isoleucine, and valine) were added up to a concentration of 10 mM each. GTP was added to a concentration of 5 mM. To reduce nonspecific binding, poly(dI-dC) (Amersham) was added to a final concentration of 40 µg/ml. Immediately after incubation for 30 min at 37˚C, samples were loaded onto an 8-10% (depending on the size of the PCR product) non-denaturing polyacrylamide gel. Gel-electrophoresis was performed initially at 100V for 60 min after which the voltage was lowered to 50V. Gels were air-dried, and X-ray films were developed and scanned after autoradiography. Intensities of free probe (amplicon) were quantified using Imagequant software (Molecular Dynamics). The K_d was calculated by interpolation. K_d is defined as the concentration of CodY at which 50% of the probe has shifted. The *psaR* promoter region was used as a negative control, since this gene is not regulated by CodY.

DNase 1 footprinting

DNA-fragments were end-labeled using the fmol DNA Cycle Sequencing System kit (Promega). Ten ng of end-labeled DNA was incubated for 30 min with 0, 2, 5, 10, 20, or 40 µM of purified CodY in 50 µl binding buffer (EMSA), after which 2 µl of DNase reaction buffer (31.3 U/ml DNase (Roche), 52 mM $CaCl_2$, and 1 mM Tris pH 7.6) was added. DNase treatment was stopped after 105 sec by the addition of 100 µl of stop-buffer (2.5 M ammonium acetate, 20 mM EDTA, and 10 µg/µl Herring sperm DNA) and DNA was precipitated by an ethanol precipitation (in the presence of 20 µg glycogen). Pellets were

washed with 70% ethanol, air dried, and resuspended in 10 µl formamide loading buffer (containing bromophenol-blue and xylene-blue). These samples were heated for 1 min at 99ºC, and applied to a preheated 8% polyacrylamide denaturing sequencing gel. Gels were air-dried, and autoradiography was performed by exposing the gel to an X-ray film.

***In vitro* pneumococcal adherence assay**

Adherence of pneumococci to epithelial cells was studied essentially as described previously (7, 27, 28). In short, monolayers of the human pharyngeal cell line Detroit 562 (ATCC CCL-138) were washed twice with 1 ml PBS. Aliquots of bacteria (grown to mid-log in Todd Hewitt Yeast-broth) stored at -80°C were thawed rapidly, harvested by centrifugation, and resuspended in RPMI 1640 medium without phenol red (Invitrogen) supplemented with 1% FCS to $1x10^7$ CFU/ml. One ml of bacterial suspension was allowed to adhere for 2h, after which non-adherent bacteria were removed by three washes with 1 ml PBS. For quantification of adherence, epithelial cells were subsequently detached by treatment with 25% Trypsin, 1 mM EDTA in PBS and lysed by the addition of ice-cold 0.025% Triton X-100 in PBS. Serial 10-fold dilutions were plated on blood agar plates to count the number of adherent bacteria, and corrected mathematically to account for small differences in count in the initial inoculum. Wild-type and mutant strains grew comparably in RMPI medium without Detroit 562 cells.

Experimental mouse models

Nine-week old female outbred CD-1 mice (Harlan, Horst, Netherlands) were used for all infection models. Prior to infection, D39 wild-type and *ΔcodY* were passaged in mice to maintain virulence as described previously (25). Cultures of *S. pneumoniae* D39 or Δ*codY* were grown to an OD_{600} of 0.3, and stored in aliquots at -80˚C in 10% glycerol. Prior to infection, these aliquots were spun down and bacteria were resuspended in sterile PBS. Mice were lightly anesthetized with 2.5% (vol/vol) isoflurane / O_2, and infected intranasally with 10^6 CFU of bacteria as described previously (24). At predetermined time points after infection, groups of mice were sacrificed by cervical dislocation and samples of various sites were taken to determine the bacterial load. In the colonization model, five mice per group were infected with 10 µl of PBS containing 10^6 CFU of either D39 wild-type or Δ*codY*, a volume small enough to only infect the nose (nasopharynx) of the mice. Bacteria were recovered from the nasopharynx by flushing the nostrils with 2 ml of sterile PBS (26), and lungs were removed from the body and homogenized in 2 ml of sterile PBS using a hand held

homogenizer (polytron PT 1200, Kinematica AG). Viable bacteria in nasal lavage fluid and homogenized lungs were counted by plating serial 10-fold dilutions on Colombia blood agar (Oxoid) supplemented with 5% (vol/vol) defibrinated sheep blood (Biotrading). Time points for sampling were 0, 24, 48, 96 and 192 h post-infection. For the pneumoniae model, five mice per group were infected with 50 µl of PBS containing 10^6 CFU of either D39 wild-type or Δ*codY*. Viable bacteria were recovered and quantified from the different sites as described above. In addition, a blood sample was removed by a cardiac puncture using a 1-ml syringe. Time points for sampling were 0, 12, 24, and 36h post-infection. In the sepsis model, six mice per group were infected in a tail vein with 10^6 CFU resuspended in 100 µl of sterile PBS. Bacteria were recovered from the blood by a lateral tail vein puncture from the same mouse at 0, 12, 24, and 36h post-infection and quantified as described above. Bacteriology results are expressed as geometric mean ± standard errors of the mean (SEM). Comparison of bacterial loads was performed using Student's *t* test. In all analyses, $p<0.05$ was considered statistically significant. All experiments were performed with approval of the Animal Experimentation Committee (DEC) of the Erasmus Medical Centre.

In vivo* expression of pneumococcal *codY

Female outbred CD-1 mice were infected with 10^7 CFU of D39 wild-type according to the pneumonia model described above. Control mice were inoculated with sterile PBS only. Forty hours post-infection, mice were sacrificed by cervical dislocation, and nasal lavage fluid (NPLF) and bronchio-alveolar lavage fluid (BALF) were collected. Upon collection of 2 ml of NPLF and BALF, 20 µl was used for determination of bacterial load, and the remaining fluid was mixed with 4 ml RNAprotect (Qiagen), and incubated for 5 min at room temperature. Bacteria were collected by centrifugation (5 min at 16,000 x g and 4°C) and pellets were snap-frozen in liquid nitrogen. RNA from NPLF and BALF was isolated using the RNeasy Kit (Qiagen) with on-column DNase treatment (Qiagen). Subsequently, 200-250 ng of total RNA was amplified using the SenseAmp Kit (Genisphere). The product of this amplification was reverse transcribed by Superscript III Reverse transcriptase (Invitrogen). To confirm the absence of genomic DNA, reactions without reverse transcriptase were performed. Of the obtained cDNA, 1 µl of a 1:2 dilution served as a template for a PCR reaction with *codY*-specific primers (Table 2). The *gyrA* (*sp1219*) amplicon was used as an internal control. Gene expression was assessed in samples obtained from three individual mice.

Accession numbers

The microarray data has been deposited in the NCBI Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE7350.

Results

The CodY regulon

To identify genes regulated by CodY, we compared the transcriptional profiles of D39 wild-type and its *codY*-mutant by DNA-microarray analysis. Western blotting of wild-type and *codY*-mutant cell lysates with anti-H_6-CodY antibodies showed that CodY is present in exponentially growing (OD_{600} of 0.1 and 0.2) wild-type D39 cells and not in D39Δ*codY* (data not shown). The microarray analysis showed that pneumococcal CodY functions mainly as a transcriptional repressor, as 43 of the 47 differentially expressed genes were found to be upregulated in the *codY*-mutant at both optical densities measured (Table 3). These included mainly genes predicted to encode proteins involved in amino acid metabolism, such as the oligopeptide permease AliA/B-Ami (*aliA sp0366*, *amiACDEF*, *sp1887-sp1891*), a putative branched-chain amino acid transporter (*liv* operon, *sp0749-sp0753*), *acuB* (*sp0754*), the *ilv* operon (*sp0445-sp0450*), and a putative operon encoding *ilvE*, a branched chain amino acid aminotransferase, two hypothetical proteins and *pcp* (*sp0856-sp0860*). Additional CodY-regulated genes involved in amino acid metabolism were *gdhA*, *asd*, and *dapA* (*sp1306*, *sp1013*, and *sp1014*, respectively). The repressed gene-set also contained genes predicted to be involved in other cellular processes, such as the *fat* locus encoding an iron transport system (*sp1869-sp1872*), that has been shown to contribute to pneumococcal virulence (8, 9) and *gapN* (*sp1119*), encoding NADP-dependent glyceraldehyde-3-phosphate dehydrogenase. Interestingly, a cluster of five genes encoding a putative transcriptional regulator, a putative bacteriocin (12), and three putative membrane proteins (*sp0141-sp0145*), was also strongly derepressed.

In addition to *codY* itself, three genes were found to be downregulated in the *codY*-mutant: *sp1429*, predicted to encode a peptidase, *sp2136,* encoding the choline binding protein PcpA, and *dpr* (*sp1572*), coding for a putative starvation-induced protein.

To examine if the transcriptional differences corresponded with changes in protein expression 2D DIGE was performed with protein isolated from D39 wild-type and Δ*codY*. Fifteen proteins were identified as being significantly more abundant in the *codY*-mutant, and of these, eight of the corresponding genes had also been identified as differentially expressed by microarray analysis (Table 3). Upregulated proteins identified solely by 2D DIGE included the penicillin binding protein PbpA (*sp0369*), heat shock protein GrpE (*sp0516*), and glucosamine-6-phosphate isomerase (*sp1415*).

Table 3. Differentially expressed genes in D39 Δ*codY*.

Gene name	ID TIGR4	ID D39	Annotation[a]	MA ratio[b]	2D ratio[c]	EMSA[d]	K_d (nM[e])	
							CodY	CodY (10nM ILV)
mutR	*sp0141*	*spd0144*	positive transcriptional regulator of MutA	2.51		+	1115	439
	sp0142	*spd0145*	hypothetical protein (bacteriocin)	2.24		+	> 2000	1938
	sp0143	*spd0146*	hypothetical protein	2.48				
	sp0144	*spd0147*	hypothetical protein	2.84				
	sp0145	*spd0148*	hypothetical protein	2.43				
	sp0159	*spd0161*	hypothetical protein	2.46		+	834	73
cps2K	-	*spd0326*	UDPglucose 6-dehydrogenase		1.47			
aliA	*sp0366*	*spd0334*	oligopeptide-binding protein	2.18	2.98			
pbpA	*sp0369*	*spd0336*	penicillin-binding protein 1A	-0.09	1.92			
ilvB	*sp0445*	*spd0404*	acetolactate synthase large subunit	3.02		+	> 2000	284
ilvN	*sp0446*	*spd0405*	acetolactate synthase small subunit	3.24				
ilvC	*sp0447*	*spd0406*	ketol-acid reductoisomerase	3.03	2.28			
	sp0448	*spd0407*	hypothetical protein	2.98				
	sp0449	*spd0408*	hypothetical protein	3.70				
ilvA	*sp0450*	*spd0409*	threonine dehydratase	2.95	1.70			
grpE	*sp0516*	*spd0459*	heat shock protein GrpE	0.22	1.26			
livJ	*sp0749*	*spd0652*	ABC transporter substrate-binding protein - BCAA transport	1.88		+	597	52
livH	*sp0750*	*spd0653*	ABC transporter membrane-spanning permease - BCAA transport	1.58				
livM	*sp0751*	*spd0654*	ABC transporter membrane-spanning permease - BCAA transport	1.90				
livG	*sp0752*	*spd0655*	ABC transporter ATP-binding protein - BCAA transport	2.25				
livF	*sp0753*	*spd0656*	ABC transporter ATP-binding protein - BCAA transport	2.30				
acuB	*sp0754*	*spd0657*	acetoin utilization protein	1.08		+	>2000	773
pnp	*sp0831*	*spd0726*	purine-nucleoside phosphorylase	0.35	1.44			
ilvE	*sp0856*	*spd0749*	branched-chain amino acid aminotransferase	2.08	[f]	+	482	68
	sp0857	*spd0750*	ABC-SBP-internal deletion, aliB-like (blastN)	2.09				
	sp0858	*spd0751*	hypothetical protein	2.15				
	sp0859	*spd0752*	membrane protein	2.07				
pcp	*sp0860*	*spd0753*	pyrrolidone-carboxylate peptidase	1.39	1.75			
	sp0882	*spr0778*	putative esterase, *S. suis* (blastN)	1.64				
	sp0884	*spd0780*	putative esterase *S. suis* (blastN)	1.78		-		
	sp0885	*spd0781*	putative Carbamoylphosphate synthase large subunit (blastN)	1.94				
asd	*sp1013*	*spd0900*	aspartate-semialdehyde dehydrogenase	2.06	1.40	+	> 2000	120
dapA	*sp1014*	*spd0901*	dihydrodipicolinate synthase	1.69				
gapN	*sp1119*	*spd1004*	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	1.05		+	752	211
glxK	*sp1126*	*spd1011*	glycerate kinase	1.33				
gdhA	*sp1306*	*spd1158*	glutamate dehydrogenase (NADP+)	1.71	2.80	+	726	156
rplL	*sp1354*	*spd1187*	50S ribosomal protein L7/L12	-0.17	2.20			
rplJ	*sp1355*	*spd1188*	50S ribosomal protein L10	-0.10	1.43			
nagB	*sp1415*	*spd1246*	N-acetylglucosamine-6-phosphate isomerase	-0.19	1.37			
	sp1578	*spd1408*	methyltransferase, putative	1.16		+	>2000	368
dctA	*sp1753*	*spd1563*	dicarboxylate/amino acid:cation (Na+ or H+) symporter	2.81				
	sp1754	*spd1564*	hypothetical protein (integral membrane protein)	2.84				

fatD	*sp1869*	*spd1649*	iron-compound ABC transporter, permease protein	2.80		+	>2000	>2000
fatC	*sp1870*	*spd1650*	iron-compound ABC transporter, permease protein	2.44				
fecE	*sp1871*	*spd1651*	iron-compound ABC transporter, ATP-binding protein	1.37				
fatB	*sp1872*	*spd1652*	iron-compound ABC transporter, iron-compound-binding protein	1.07				
amiD	*sp1889*	*spd1669*	oligopeptide ABC transporter, permease protein	1.20				
amiC	*sp1890*	*spd1670*	oligopeptide ABC transporter, permease protein	1.72				
amiA	*sp1891*	*spd1671*	oligopeptide ABC transporter, oligopeptide-binding protein	1.21	1.41	+	677	433
	sp2125	*spd1954*	hypothetical protein	2.18		+	754	630
rpoA	*sp0236*	*spd0218*	DNA-directed RNA polymerase subunit alpha	-0.12	-1.86			
ABC-NBD	*sp0483*	*spd0434*	ABC transporter ATP-binding protein - cobalt transport	-0.34	-1.96			
fba	*sp0605*	*spd0526*	fructose-bisphosphate aldolase	-0.20	-1.64			
pepV	*sp0623*	*spd0542*	dipeptidase	-0.21	-2.42			
	sp1429	*spd1258*	putative peptidase, U32 family	-1.29	-1.81			
pgm	*sp1498*	*spd1326*	phosphoglucomutase	0.11	-1.57			
atpB	*sp1513*	*spd1340*	proton-translocating ATPase, F0 sector, subunit a	-0.15	-1.58			
dpr	*sp1572*	*spd1402*	DNA binding protein starved cells-like peroxide resistance protein	-2.69	-1.55			
codY	*sp1584*	*spd1412*	transcriptional pleiotropic repressor CodY	-1.68		+	> 2000	382
gapA	*sp2012*	*spd1823*	glyceraldehyde-3-phosphate dehydrogenase	-0.20	-1.93			
pcpA	*sp2136*	*spd1965*	choline binding protein PcpA	-1.36		+	> 2000	903
pgsA	*sp2222*	*spd2049*	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	0.14	-1.67			

[a] Annotation is according to the Kyoto Encyclopedia of Genes and Genomes database (www.kegg.com).

[b] Microarray (MA) ratios are given as follows: expression of Δ*codY* / expression of wild-type (Log_2 transformed)

[c] 2D DIGE (2D) ratios are given as: expression of Δ*codY* / expression of wild-type (Log_2 transformed)

[d] In the EMSA column, a plus indicates a shift, while a minus indicates no shift.

[e] The K_ds of the promoter region (concentration of CodY at which 50% of the probe is shifted) without addition of BCAAs and in the presence 10 mM BCAAs are shown.

[f] Only expressed in the *codY*-mutant.

Ten proteins were found to be significantly more abundant in the wild-type than the mutant, two of which identified by transcriptional analysis as well (Table 3). Proteins only detected by 2D-DIGE included fructose biphosphate aldolase (Fba*, sp0605*), glyceraldehyde-3-phophate dehydrogenase (GapA, *sp2012*), F-type H+-transporting ATPase a chain (AtpB, *sp1513*), and the dipeptidase PepV (*sp0623*). Interestingly, *L. lactis* PepV belongs to the proteolytic system regulated by CodY, although direct regulation of *pepV* by CodY has never been shown (16).

Binding of CodY to target promoters

To discriminate between direct and indirect regulation by CodY, electrophoretic mobility shift assays (EMSA) were performed. Upstream regions containing putative promoter regions of genes identified by either the microarray analysis, 2D DIGE analysis, or *in silico* screening using the *L. lactis* binding consensus sequence (AATTTTCWGAAAATT, (14)) were amplified and incubated with purified H_6-CodY. Also, the effect of addition of branched chain amino acids (BCAAs) and GTP on DNA-binding to these promoters was investigated. The upstream region of *codY* was bound by purified H6-CodY especially in the presence of BCAAs, suggesting that CodY regulates its own expression (Table 3). Five promoter regions of genes or operons predicted to be involved in BCAA biosynthesis or transport showed a shift, namely P*ilvB*, P*ilvE*, P*livJ*, P*amiA* (Table 3), and P*aliB* (in microarray analysis it was 1.8-fold upregulated in Δ*codY*). This binding of CodY was enhanced by addition of BCAAs with a factor 2 to 20. For instance, the K_d (CodY-concentration at which 50% of the DNA-probe is shifted) of P*ilvE* and P*livJ* ranged from 480-600 nM CodY without the addition of BCAAs, and 50-70 nM CodY in the presence of BCAAs (Table 3). No effect of GTP on DNA-binding was observed (data not shown). Binding of CodY to the other three promoters was also enhanced by BCAAs, although to a lower extent. Other promoter regions to which CodY was able to bind were P*gdhA*, P*fatD*, P*acuB*, P*asd*, P*gapN*, P*sp0141*, P*sp0142*, P*sp0159*, P*sp1578*, and P*sp2125* (Fig. 1A and Table 3). Several of these promoters had a K_d (without BCAAs) higher than the highest CodY concentration tested (Table 3), indicating that their K_d was greater than 2000 nM. Again, the affinity of CodY for the promoters was enhanced by the presence of BCAAs (K_d ranging from 150-1950 nM, depending on the promoter region). All but two of these promoter regions (P*sp0141* and P*sp0142*) contained a sequence resembling the CodY binding box. Finally, CodY also bound to the upstream region of *pcpA*, one of the genes downregulated in the *codY*-mutant, and this binding was enhanced by addition of BCAAs (Table 3). In addition, a sequence resembling the CodY-box is present 105 bp upstream of the *pcpA* startcodon (5'-AATTTATAAAATGTA-3'). This suggests that CodY might positively regulate the expression of PcpA, a choline binding protein suggested to be involved in adherence (37).

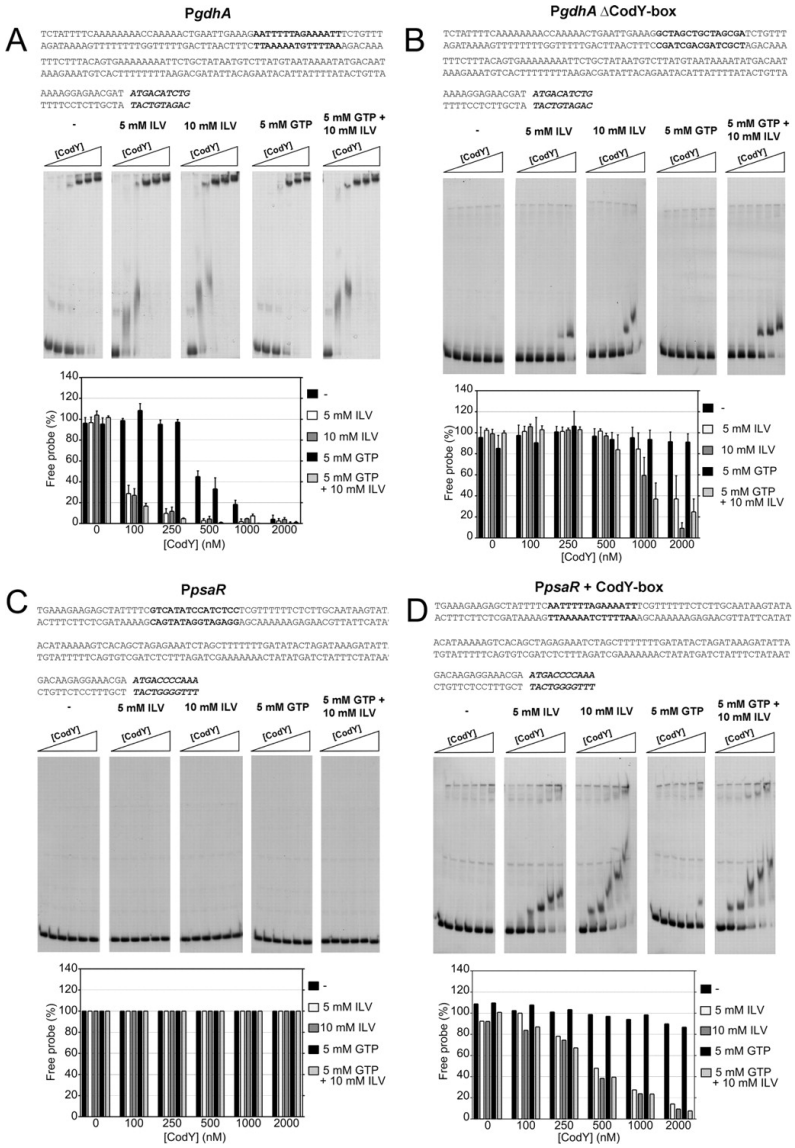

Figure 1. Electrophoretic Mobility Shift Assay (EMSA) using H6-CodY. DNA-binding of CodY to the *gdhA*-promotor region (140 bp) and the *psaR*-promoter region (147 bp) in the absence or presence of BCAA (5 or 10 mM ILV) and/or GTP (5mM). In each series, successive lanes contained increasing amounts of CodY (0, 100, 250, 500, 1000 and 2000 nM). In the sequences (top), bold font indicates the CodY-recognition site (or its position in the mutated sequence) and the start of coding sequence is indicated in bold and italics. Histograms (bottom) show the percentage of free probe (DNA-fragment) left. **(A)** The wild-type *gdhA*-promotor region, **(B)** the mutated *gdhA*-promoter with the CodY-box replaced with random nucleotides, **(C)** the wild-type *psaR*-promoter, not regulated by CodY and, **(D)** the mutated *psaR*-promoter, with a CodY-box introduced 100 bp upstream of the coding sequence.

EMSA of the *gdhA* promoter revealed the presence of two retarded protein-DNA complexes, a major one with lowest mobility seen at intermediate to high concentrations of CodY, and one with intermediate mobility seen with lower concentrations of CodY only in the presence of BCAAs (Fig. 1A). Similar patterns were observed for other promoters tested (not shown). To gain further insight into the binding of CodY to the *gdhA* promoter region, the sequence matching the *L. lactis* consensus sequence was replaced with random nucleotides and tested by EMSA (Fig. 1B). The distance of this consensus sequence to the startcodon is 79 bp. Without its putative binding domain, CodY did bind the DNA, but only at high concentrations, i.e., 1000 nM and 2000 nM. Furthermore, only the intermediately shifted band was seen. The major complex might consist of the promoter region to which two (or more) CodY dimers have bound, one to the CodY-box, and one to a secondary binding site (Fig. 1A). This putative secondary binding site would still be present in the mutated *gdhA* promoter fragment, resulting in an intermediate shift. Next, the upstream sequence of the gene *psaR* (*sp1638*), to which CodY is not able to bind under any condition (Fig. 1C), was mutated in such a way that the CodY-box consensus sequence was introduced. Although no CodY binding to this DNA-fragment was observed without BCAAs, a clear shift was observed upon addition of BCAAs (K_d of 400 nM), indicating that this sequence is indeed involved in protein-DNA interaction (Fig. 1D). Only a complex with intermediate mobility was seen, indicative of binding of one CodY dimer to the introduced CodY-box. In comparison to the P*gdhA* with CodY-box, this intermediate shift occurs at lower CodY concentrations, suggesting that CodY has a higher affinity for the CodY-box than for the putative secondary binding site.

DNase 1 Footprinting of the *gdhA* and *codY* promoters

To determine the CodY binding sites in more detail, we performed DNase I footprint assays. First, H_6-CodY was incubated with a 160-bp DNA-fragment containing the promoter region of *codY*, both without and with the addition of BCAAs (10 mM ILV) or BCAAs (10 mM ILV) and GTP (5 mM). Increased protection from DNase1 was observed with increasing concentrations of H_6-CodY (Fig. 2A). This protection was not enhanced by addition of GTP (data not shown) or, surprisingly, BCAAs (Fig. 2A). Three protected regions were identified, one of which contained the putative CodY-box (region I, Fig. 2A).

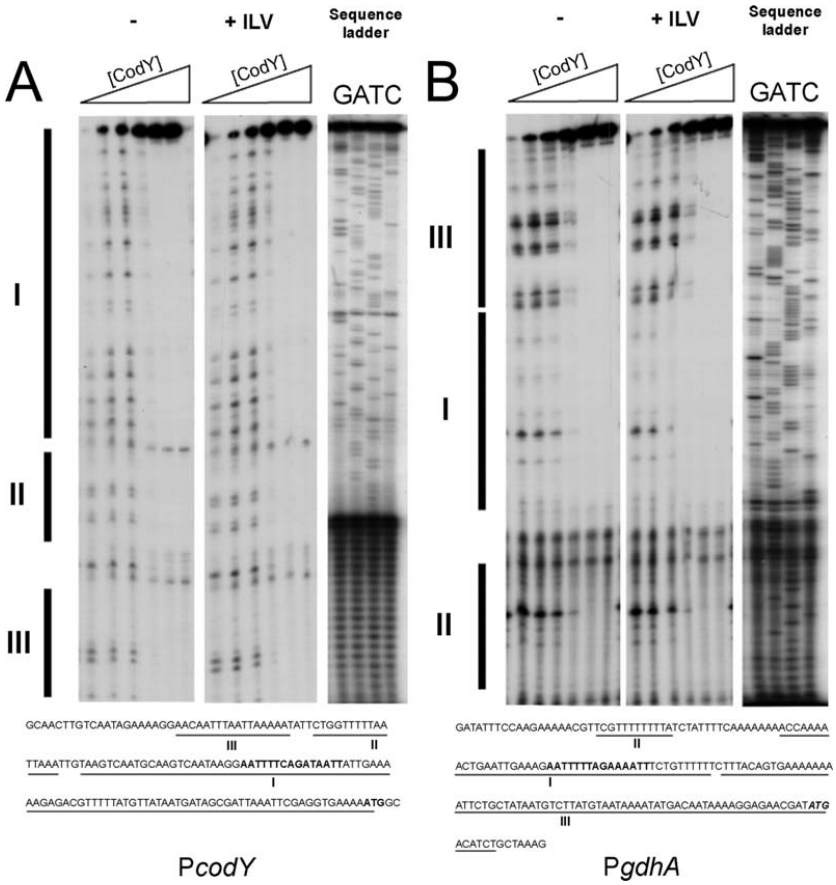

Figure 2. DNase1 Footprinting using H_6-CodY. DNase1 footprint of the **(A)** *codY*- and **(B)** *gdhA*-promotor regions, in the absence or presence of BCAA (10 mM ILV). In each series, successive lanes contained increasing amounts of CodY (0, 2, 5, 10, 20 and 40 μM). Protected regions are marked by vertical bars and underlined in the DNA sequence (bottom). The CodY-boxes are given in bold fonts and the startcodons are given in italic and bold fonts.

Additional protected sequences were found to be AT-rich DNA sequences (region II and III, Fig. 2A). Next, the 178-bp region upstream of *gdhA* was incubated with H_6-CodY. Again, we did not observe any effect of addition of BCAA (Fig. 2B) or GTP (data not shown). Three regions were protected from DNase 1 degradation. One large region, similar to that observed in the *codY* promoter region, contained the putative CodY-box (region I, Fig. 2B). The second region was a T-rich domain (region II, Fig. 2B). The third region was an additional region downstream of region I. At higher CodY concentrations region I and III appeared to form a large protected DNA sequence of approximately 120 bp (Fig. 2B). Apart from the CodY-box, the *codY* and *gdhA* promoter regions bear no clear similarities.

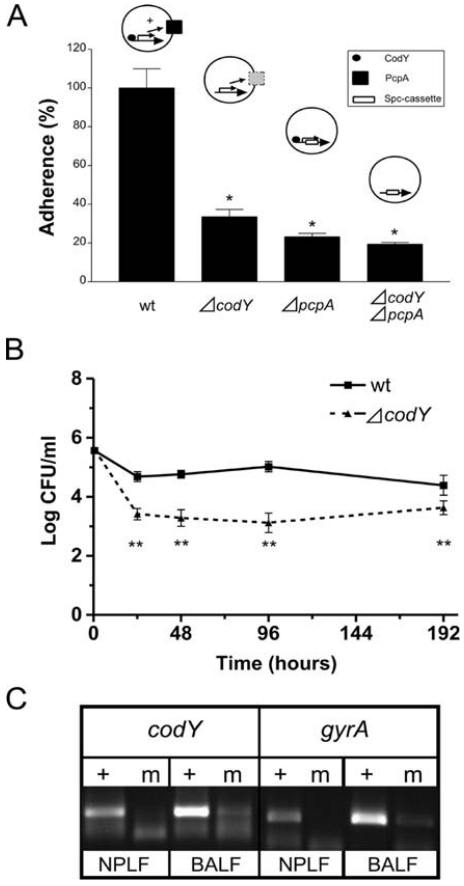

Figure 3. Involvement of CodY in adherence and colonization. **(A)** *In vitro* adhesion of D39Δ*cps*, D39Δ*cps*Δ*codY*, D39Δ*cps*Δ*pcpA*, and D39Δ*cps*Δ*codY*Δ*pcpA* to Detroit 562 nasopharyngeal cells. The adherence of the *codY*-mutant is given as percentage relative to the wild-type. A possible model for regulation of *pcpA* by CodY is given above the histogram. For explanation see text. **(B)** Intranasal challenge with 10^6 CFU of either D39 wild-type or D39Δ*codY*. **(C)** Expression of *codY* during experimental virulence. Transcripts of *codY* and *gyrA* were identified in bacterial RNA isolated from nasopharyngeal lavage fluid (NPFL) and bronchio-alveolar lavage fluid (BALF) 40 hours post-infection. + indicates a reaction with reverse transcriptase, and m indicates the negative control (without reverse transcriptase). * p <0.0001 (Mann Whitney U test). ** p <0.05 (Student's t test).

Adherence of the *codY*-mutant to pharyngeal cells

Colonization of the nasopharynx is mediated by adherence of the bacterium to respiratory epithelial cells, a process that likely needs to be tightly regulated. To examine the

role of CodY in this process, we tested the ability of D39Δ*codY* to adhere to the human pharyngeal epithelial cell line Detroit 562 *in vitro*. Since encapsulated strains tend to adhere to a lower extent compared to unencapsulated strains, a mutant for *codY* in a capsule-negative genetic background (D39Δ*cps*) was constructed (7). Clearly, D39Δ*cps*Δ*codY* was less capable to adhere to human pharyngeal cells than D39Δ*cps* ($p<0.0001$, Fig. 3A). As mentioned, *pcpA*, encoding a putative adhesin (37), was found to be downregulated in the *codY* mutant, meaning that this gene is activated by CodY. To investigate the role of CodY activation on adherence through PcpA, we constructed a D39Δ*cps*Δ*pcpA* strain and a D39Δ*cps*Δ*codY*Δ*pcpA* strain and tested their ability to adhere to the human epithelial cells. D39Δ*cps*Δ*pcpA* showed significantly lower levels of adherence than D39Δ*cps* ($p< 0.0001$, Fig 3A). This difference was even greater than that between the D39Δ*cps* and D39Δ*cps*Δ*codY* strains (Fig. 3A).

Contribution of CodY to experimental virulence

To assess the contribution of *codY* to pneumococcal virulence, D39 wild-type and D39Δ*codY* were tested in three mouse models of pneumococcal infection. No significant differences in bacterial loads were observed using the pneumonia and sepsis model of infection (data not shown). In the colonization model of infection, however, a clear phenotype for D39Δ*codY* was seen: a consistently and significantly lower bacterial load compared to the wild-type strain for the duration of the infection ($p<0.0015$, Fig. 3B).

To assess whether *codY* is actually expressed at the different sites during infection, we isolated bacterial RNA from the nasopharyngeal and bronchio-alveolar lavage fluid obtained from mice infected with D39 wild-type. Indeed, a clear *codY* transcript could be detected in the nasopharynx and lungs at 40 hours post-infection (Fig. 3C).

Discussion

CodY has been described as a nutritional repressor in various bacteria, in which it represses genes that are involved in biosynthesis and uptake of amino acids, as well as genes that are typically expressed during late exponential or stationary phase (14, 35). The aim of the present study was to elucidate the role of CodY in the physiology of *S. pneumoniae*. Transcriptome and proteome analyses identified several genes previously shown to be part of the CodY-regulon in other bacteria (Table 3). Ten of the genes and proteins were identified as CodY targets by both techniques. Discrepancies observed between transcriptional and proteome analysis could be due to several reasons, such as low levels of gene expression, instability of proteins, or specific regulation at the translational level.

The pneumococcal CodY-regulon predominantly consists of genes and operons involved in BCAA metabolism and general amino acid metabolism, such as the *ilv* operon (*ilvBNC*) and the genes *ilvA* and *ilvE* which were found to be strongly upregulated in the *codY*-mutant. Interestingly, *ilvA*, *ilvD*, and *ilvE* are strongly upregulated in a ciprofloxacin-resistant *S. pneumoniae* strain compared to its ciprofloxacin-sensitive parental strain after induction with this antibiotic (34). However, both D39 wild-type and the *codY*-mutant were found to be sensitive for ciprofloxacin suggesting that these enzymes are not directly involved in ciprofloxacin resistance (data not shown). In *B. subtilis*, the *ilvBNC* operon is tightly regulated by three regulators, CcpA, TnrA and CodY. These regulators can activate or repress transcription of the *ilv* operon, depending on nutritional conditions (47). No TnrA homologue is found in the genome of the pneumococcus and regulation of *ilvBNC* by CcpA has not been investigated yet in *S. pneumoniae*. Another example of a gene whose expression is controlled by multiple regulators is *gdhA* (glutamate dehydrogenase). Apart from its repression by CodY, the expression of this gene is also regulated by the nitrogen regulatory protein GlnR (28), suggesting that GdhA plays a central role within pneumococcal nitrogen metabolism.

Preliminary results indicate that the intracellular amino acid pool is indeed affected in the *codY*-mutant: lower intracellular glutamate and higher NH_3 concentrations were measured (unpublished results). This is probably due to the higher abundance of GdhA, which catalyzes the deamination of glutamate into alpha-ketoglutarate and NH_3. The higher NH_3-concentration may also be the result of the higher abundance of threonine dehydratase (IlvA), as this enzyme catalyzes the conversion of threonine to 2-oxobutanoate and NH_3. Accordingly, the butanoate concentration was also higher in the *codY*-mutant (unpublished results).

Using DNAse1 footprinting, we identified the pneumococcal CodY-binding box, similar to the consensus sequence described for *L. lactis.* Furthermore, EMSA showed that most of the genes upregulated in the *codY*-mutant are under direct control of CodY, although CodY does not have the same affinity for all promoters. Like in *L. lactis*, BCAAs but not GTP enhanced binding of H_6-CodY to its target promoters. For instance, the K_d without BCAAs for P*codY* was >2000 nM, whereas with BCAAs it was 382 nM. This suggests that when high concentrations of BCAAs are present, CodY might repress itself stringently, whereas the affinity of CodY for its own promoter is relatively low in the presence of low amounts of BCAAs. On the other hand, the overall affinity of CodY for the *gdhA*-promoter region was much higher, indicating that even in very low BCAA concentrations CodY might still able to repress *gdhA*-expression.

CodY-targets whose expression was affected most in the *codY*-mutant (6-10 fold upregulation) were genes encoding products predicted to be involved in BCAA metabolism. Their promoter regions also displayed the highest affinity for CodY, especially in the presence of BCAAs. Among these was the previously-mentioned *ilvBNC* operon, which encodes enzymes that condense threonine and pyruvate or two pyruvates into branched-chain keto-acids, precursors of the BCAAs. Derepression of this operon might therefore result in an alteration of the pyruvate pool (40). Previous studies have shown that a mutant for pyruvate oxidase, encoded by *spxB*, is affected in its ability to adhere to type II lung cells and epithelial cells (43). It was found that upon addition of acetate, adherence of the *spxB*-mutant was restored to wild-type levels, indicating that SpxB is not an adhesin and that acetyl-CoA influences the adhesive properties of pneumococci. Metabolites, which pneumococcus produces during colonization, could potentially play a role in creating a favorable environment for adhesion. Normally, bacteria use the transport of acids like acetate and lactate out of the cell to build up an electrochemical gradient (proton motive force). As a result of the *codY* mutation, *S. pneumoniae* might no longer be able to adequately maintain this proton motive force. From a nutritional point of view, without cellular active CodY, the cell is considered to be in a "hunger state", which is in agreement with the hypothesis of Spellerberg and co-workers, who proposed that pneumococcus adheres in a nutrient-rich, but not in a nutrient-poor environment (43). This suggests that adhesins are preferentially expressed in nutrient-rich conditions. In line with this, expression of the choline binding protein PcpA, a putative adhesin (37), was downregulated in the *codY*-mutant, and CodY bound to the *pcpA* upstream region suggesting a possible link between nutritional regulation and adhesion. Our *in vitro* adhesion assays showed that PcpA is indeed required for wild-type

levels of adherence in D39. Using a *cps-codY-pcpA* triple mutant, we showed that there is an additional effect when *pcpA* is deleted in a *codY*-mutant. This could indicate that in the case of a *codY*-mutation, no induction of *pcpA*-transcription occurs, but there is still some background transcription allowing sub-optimal adherence compared to the isogenic wild-type. The *cps-codY-pcpA* triple mutant adhered at comparable levels as the *cps-pcpA* mutant, suggesting that induction of *pcpA*-expression by CodY might be required for efficient adherence to Detroit 562 cells. In this way expression of adhesins might be controlled by nutritional regulation (Fig. 3A). However, PcpA was shown not to be required for colonization in another strain (22), so the observed effect of the *codY*-mutation on adherence and colonization could be indirect. In contrast, the oligopeptide permease AliA/B-Ami complex, strongly upregulated in the *codY*-mutant, has been shown to be either directly or indirectly involved in interaction with type II lung cells and epithelial cells (11). Furthermore, an *aliA/B-ami* mutant has been shown to be attenuated for colonization in a murine model of infection (25). Because the *codY*-mutant was also attenuated for colonization, while overexpressing AliA/B-Ami, it is likely that this oligopeptide permease is indirectly involved in adherence and colonization (i.e., by modulating adhesins), although further experiments are needed to verify this.

By identifying the pneumococcal CodY-regulon, we were able to confirm its role as a nutritional regulator described for other Gram-positives, and at the same time show species-specific targets, such as the putative bacteriocin system. In contrast to *B. subtilis*, no genes directly involved in competence appeared to be under control of CodY. The only link between CodY and competence is the regulation of the oligopeptide permease AliA/B-Ami, which has been indirectly implicated with the development of competence (10). Furthermore, we have shown that CodY is required for the colonization of the nasopharynx, in particular through adherence to epithelial cells as demonstrated *in vitro*. However, it remains as yet unclear which factor(s) (adhesins, proton motive force, intracellular or extracellular metabolites) are the main players during colonization and adhesion.

In conclusion, the CodY-regulon of *S. pneumoniae* is of profound importance for the adaptation of this bacterium to nutrients. As such, this regulon is considered to contribute to the early stages of infection, i.e., colonization of the nasopharynx.

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CHAPTER 5

Regulation of glutamine and glutamate metabolism by GlnR and GlnA in *Streptococcus pneumoniae*

Nitrogen Metabolism in *Streptococcus pneumoniae*

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Abstract

Several genes involved in nitrogen metabolism are known to contribute to the virulence of pathogenic bacteria. Here, we studied the function of the nitrogen regulatory protein GlnR in the Gram-positive human pathogen *Streptococcus pneumoniae*. We demonstrate that GlnR mediates transcriptional repression of genes involved in glutamine synthesis and uptake (*glnA*, *glnPQ*), glutamate synthesis (*gdhA*), and the gene encoding the pentose phosphate pathway enzyme Zwf, which forms an operon with *glnPQ*. Moreover, the expression of *gdhA* is also repressed by the pleiotropic regulator CodY. The GlnR-dependent regulation occurs through a conserved operator sequence and is responsive to the concentration of glutamate, glutamine and ammonium in the growth medium. By means of *in vitro* binding studies and transcriptional analyses we show that the regulatory function of GlnR is dependent on GlnA. Mutants of g*lnA* and *glnP* displayed significantly reduced adhesion to Detroit 562 human pharyngeal epithelial cells, suggesting a role for these genes in the colonization of the host by *S. pneumoniae*. Thus, our results provide a thorough insight into the regulation of glutamine and glutamate metabolism of *S. pneumoniae* as mediated by both GlnR and GlnA.

Introduction

Regulation of nitrogen metabolism in bacteria is closely connected with the intracellular levels of glutamine and glutamate, the main nitrogen donors in the cell. Glutamine is formed from glutamate and ammonium by glutamine synthetase (GlnA), which is a major way for the cell to assimilate ammonium. Glutamate can be formed either by glutamate dehydrogenase from 2-oxoglutarate and ammonium, or by glutamate synthase, which converts glutamine and 2-oxoglutarate into two molecules of glutamate.

Several studies indicate that nitrogen metabolism, especially glutamine metabolism, is important for the virulence of various bacterial pathogens (24, 43, 45)Signature-tagged mutagenesis screens suggest that genes involved in glutamine metabolism, *glnQ* and *glnA*, are likely to play a role in the virulence of *S. pneumoniae* as well (18, 28, 36). However, so far, glutamine metabolism and the way in which it is regulated have not been studied in this human pathogen.

In the well-characterized Gram-positive bacterium *Bacillus subtilis*, regulation of nitrogen metabolism is carried out mainly by CodY, GlnR and TnrA (13). The latter two are members of the MerR family of regulators, and both recognize the same operator sequence: '5-TGTNAN$_7$TNACA-3'. TnrA functions during growth on a poor nitrogen source, for example solely glutamate, when it activates or represses expression of various genes involved in nitrogen metabolism (5, 32, 41, 50, 53). GlnR represses its own operon *glnRA* (8), the *ureABC* operon (encoding urease) (7, 49) and *tnrA* (13) in the presence of a good nitrogen source, like glutamine.

Genetic experiments have shown that genes regulated by GlnR and TnrA are constitutively expressed in a mutant of *glnA* (38-40, 50). An explanation for this observation came with the discovery that *in vitro* DNA-binding by TnrA is blocked by feedback-inhibited GlnA (52). Although it has been suggested that GlnA also controls the DNA-binding activity of GlnR, this has never been shown. In fact, *B. subtilis* GlnR has a high affinity for DNA on its own (8).

B. subtilis CodY functions as a repressor of genes involved in nitrogen metabolism (20), but also of carbon and energy metabolism (22), motility (6) and competence development (31). In the lactic acid bacterium *Lactococcus lactis*, CodY represses genes of the proteolytic system and several amino acid transport and metabolism genes, amongst others *gltA* and *gltD*, which are involved in glutamate biosynthesis (11, 15, 16).

Analysis of the *S. pneumoniae* R6 (19) and TIGR4 (44) genomes revealed that they contain genes encoding orthologs of GlnR and CodY, but not of TnrA. Furthermore, *S.*

pneumoniae contains a putative ortholog of *glnA*, several predicted glutamine uptake systems and a predicted biosynthetic glutamate dehydrogenase (19, 44). In contrast to *B. subtilis* and *L. lactis*, a gene encoding glutamate synthase is not present. This suggests that *S. pneumoniae* has various ways to warrant sufficient cellular glutamine levels, either by uptake from the environment or by *de novo* synthesis.

In this study, we report on the important role of GlnR and GlnA in the regulation of glutamine and glutamate metabolism in *S. pneumoniae*, and present indications for a role of GlnR-targets in pneumococcal virulence.

Materials and Methods

Strains, media and growth conditions

Strains used in this study are listed in Table 1 and were stored in 10% glycerol at -80ºC. *S. pneumoniae* was grown essentially as described (23): on plates in a flame-pot, giving an elevated CO_2 concentration, or in liquid medium as standing cultures. *L. lactis* and *E. coli* were grown as described previously (23). Kanamycin and tetracycline were used in concentrations of 500 μg/ml and 2.5 μg/ml for *S. pneumoniae*, respectively. Ampicillin was used in a concentration of 100 μg/ml for *E. coli*. Chemically defined medium with a final pH of 6.4 was composed as described (23), except that sodium-citrate was used in the buffer instead of ammonium-citrate and that glutamine was omitted from the amino acid mixture. Glutamine, glutamate and ammonium were added as specified in the Results section. Induction of gene expression with nisin was performed as described, using a stock solution of nisaplin, containing 20 mg/ml nisin (23).

DNA isolation and manipulation

Primers used in this study are listed in Table 2. Primers were based on the genome sequence of strain *S. pneumoniae* R6 (19). Unless otherwise indicated, chromosomal DNA of *S. pneumoniae* D39 was used as a template for PCR amplification. All DNA manipulations were done as described (23).

Construction of *glnR, glnA, glnRA*, *zwf* and *glnP* mutants of *S. pneumoniae*

The *glnR*-stop mutant (TK102) was constructed using plasmid pORI280 as follows. Primer glnR-stop 1 with two point mutations, leading to two premature stop codons at codon-positions 20 and 21 in the *glnR* reading frame, was used in combination with primer glnR_R6-1 to PCR amplify a fragment comprising the upstream part and the beginning of *glnR*. A second PCR product, comprising the rest of the *glnR* gene and part of the downstream sequence, was produced with primers glnR-stop 2 and glnR-3. These PCR products were complementary by 20-bp covering the position of the stop codons and were used as a template in a PCR reaction with primers glnR_R6-1 and glnR-3. The resulting product was cloned as an *Xba*I, *Bgl*II fragment in pORI280, giving plasmid pTK20. pTK20 was used to introduce the mutations into the chromosome of *S. pneumoniae* D39 as described (23), giving strain TK102. The mutations led to the disappearance of a *Hinc*II site, on the basis of which the proper mutant could be identified. The mutations were further verified by DNA sequencing.

Table 1. Strains and plasmids used in this study.

Strain/plasmid	Description	Reference or source
S. pneumoniae		
D39	Serotype 2 strain, *cps2*	(2), lab. P. Hermans
D39*repA*	D39 Δ*bgaA::repA*; $Trmp^R$	(23)
D39*nisRK*	D39 Δ*bgaA::nisRK*; $Trmp^R$	(23)
TK100	D39*nisRK* Δ*glnA*; $Spec^R$	This work
TK102	D39 *glnR*-stop, contains two stop mutations in the start of *glnR*	This work
TK103	D39 Δ*glnA*; $Spec^R$	This work
TK104	D39 Δ*glnRA*; $Spec^R$	This work
TK105	TK102 Δ*bgaA::nisRK*; $Trmp^R$	This work
TK106	D39 Δ*glnP*; Em^R	This work
TK107	D39 Δ*zwf*; $Spec^R$	This work
TK108	D39 Δ*gdhA*; Em^R	This work
TK109	TK102 Δ*bgaA::repA*; $Trmp^R$	This work
WH101	D39 Δ*codY*; $Trmp^R$	This work
TK108	TK102 Δ*codY*; $Trmp^R$	This work
TK109	TK106 Δ*bgaA::nisRK*; $Trmp^R$	This work
TK110	D39*nisRK glnA-lacZ*; Em^R	This work
TK111	D39*nisRK* P*glnP-lacZ*; Em^R	This work
TK112	D39*nisRK gdhA-lacZ*; Em^R	This work
TK113	D39*nisRK zwf-lacZ*; Em^R	This work
TK114	D39*nisRK* P*arcA-lacZ*; Em^R	This work
TK120	TK105 *glnA-lacZ*; Em^R	This work
TK121	TK105 P*glnP-lacZ*; Em^R	This work
TK122	TK105 *gdhA-lacZ*; Em^R	This work
TK123	TK105 *zwf-lacZ*; Em^R	This work
TK125	TK105 P*arcA-lacZ*; Em^R	This work
TK126	TK100 *glnA-lacZ*; Em^R	This work
TK127	TK100 P*glnP-lacZ*; Em^R	This work
TK129	TK100 *gdhA-lacZ*; Em^R	This work
TK130	TK100 *zwf-lacZ*; Em^R	This work
TK131	TK100 P*arcA-lacZ*; Em^R	This work
TK132	D39 Δ*bgaA*::P*gdhA*1-*lacZ*; Tet^R	This work
TK133	D39 Δ*bgaA*::P*gdhA*1-*lacZ*; Tet^R	This work
TK134	TK102 Δ*bgaA*::P*gdhA*2-*lacZ*; Tet^R	This work
TK135	TK102 Δ*bgaA*::P*gdhA*2-*lacZ*; Tet^R	This work

TK136	D39 Δ*cps*; Km^R capsule-less derivative	This work
TK137	TK102 Δ*cps*; Km^R capsule-less derivative	This work
TK138	TK103 Δ*cps*; Km^R capsule-less derivative	This work
TK139	TK104 Δ*cps*; Km^R capsule-less derivative	This work
TK140	TK106 Δ*cps*; Km^R capsule-less derivative	This work
L. lactis		
NZ9000	MG1363Δ*pepN*::*nisRK*	(27)
LL108	MG1363 $RepA^+$ (multi-copy); Cm^R	(29)
E. coli		
EC1000	Km^R; MC1000 derivative carrying a single copy of the pWV01 *repA* gene in *glgB*	(30)
Plasmids		
pORI13	Em^R; ori^+ $repA^-$; promoterless *lacZ*, for single-copy chromosomal *lacZ* fusions.	(37)
pORI280	Em^R; ori^+ $repA^-$; deletion derivative of pWV01; constitutive *lacZ* expression from P32 promoter	(30)
pORI28*	Em^R; ori^+ $repA^-$; deletion derivative of pWV01	(30)(
pORI38*	$Spec^R$; ori^+ $repA^-$; deletion derivative of pWV01	(30)
pNZ8048	Cm^R; Nisin-inducible P*nisA*	(9)
pNG8048E	Cm^R Em^R; Nisin-inducible P*nisA*, pNZ8048 derivative containing em^R gene to facilitate cloning	Laboratory collection
pPP2	Amp^R Tet^R; promoter-less *lacZ*. For replacement of *bgaA* (*spr0565*) with promoter-*lacZ* fusions. Derivative of pPP1.	(25)
pORI28spec1*	pORI28 containing $spec^R$ gene from pORI38	This work
pTK8	pORI13 '*glnA-lacZ*	This work
pTK9	pORI13 P*glnP-lacZ*	This work
pTK10	pORI13 '*gdhA-lacZ*	This work
pTK11	pORI13 '*zwf-lacZ*	This work
pTK12	pORI13 P*arcA-lacZ*	This work
pTK13	pPP2 PgdhA-1-*lacZ*	This work
pTK14	pPP2 PgdhA-2-*lacZ*	This work
pTK15	pNG8048E carrying *His6-glnA* downstream of P*nisA*	This work
pTK16	pNG8048E carrying *His6-glnR* downstream of P*nisA*	This work
pTK17	pNG8048E carrying *glnPQ* downstream of P*nisA*	This work
pTK18	pORI28spec1 containing 886 bp downstream of *glnA*	This work
pTK19	pORI28spec1 Δ*zwf*	This work
pTK20	pORI280, containing *glnR* with stop mutations (K20(AAG)→stop(**T**AG) and L21(TTG)→stop (T**A**G))	This work

pTK21	pORI13 P*zwf-lacZ*	This work
pTK22	pORI28spec1 containing 883 bp upstream sequence of *glnR*	This work
pTK23	pNG8048E carrying *glnR* downstream of P*nisA*	This work

Trmp^R, trimethoprim-resistance; Spec^R, spectinomycin-resistance; Em^R, erythromycin resistance; Tet^R, tetracycline resistance; Km^R, kanamycin-resistance; Cm^R, chloramphenicol resistance. *Plasmid sequences are available at http://molgen.biol.rug.nl/publication/glnRAspn_data**.**

The *glnA* deletion strain (TK103) was generated by allelic-replacement mutagenesis, removing 1300 bp of the *glnA* ORF: A PCR fragment, generated with primers Spec_pORI38-Fp and Spec_pORI38-Rp on the spectinomycin resistance gene from pORI38, was cloned into the *Hind*III site of pORI28 in the same orientation as the erythromycin gene on this vector, yielding pORI28spec1. Next, the 3' flanking region of *glnA*, amplified with primer pair glnA_R6-3/glnA_R6-4 (886 bp), was cloned into the *Nco*I/*Bgl*II sites of pORI28spec1, giving pTK18. pTK18 was cut with *Nde*I/*Aat*II and a PCR fragment generated with primers glnA_R6-1/glnA_R6-2 (808 bp), which was digested with the same enzymes, was ligated to it. This ligation mixture was used to generate a PCR product with primers glnA_R6-1 and glnA_R6-4, which was transformed to *S. pneumoniae* D39. Spectinomycin resistant clones were examined for the presence of the *glnA* deletion by PCR and Southern blotting. The *zwf* deletion mutant (TK107), removing 1416 bp of the *zwf* ORF, was constructed in a similar way as the *glnA* mutant, using primers G6PDH-4/G6PDH-5 (660 bp) and G6PDH-6/G6PDH-7 (610 bp).

To construct the *glnRA* mutant (TK104), the upstream part of *glnR*, amplified with primer pair glnR_R6-1/glnR_R6-2 (883 bp), was cloned into the *Xba*I/*Bam*HI sites of pORI28spec1, giving pTK22. pTK22 was used together with pTK18, which contains the glnA_R6-3/glnA_R6-4 PCR product cloned into the *Nco*I/*Bgl*II sites of pORI28spec1, as a template in a PCR reaction with primers glnR_R6-1 and glnA_R6-4. In this way a PCR product was obtained containing the spectinomycin resistance gene flanked by the upstream and downstream sequence of *glnRA*. The resulting PCR product was transformed to D39. The deletion was confirmed by PCR and Southern blotting.

L. lactis 108 was used as the cloning host for plasmid pTK19. All other construct were made in *E. coli* EC1000.

To construct the *glnP* deletion mutant (TK106), removing 2080 bp of the *glnP* ORF, a PCR fragment, generated with primer pair Ery-rev/Ery-for on the erythromycin resistance

gene from pORI28, was fused to the flanking regions of *glnP*, which were PCR amplified with primer pairs glnPKO-1/glnPKO-2 (628 bp) and glnPKO-3/glnPKO-4 (610 bp), by means of overlap-extension PCR (42). The resulting PCR product was transformed to *S. pneumoniae* D39, and clones were checked for the presence of the mutation by PCR. In the same way a deletion mutant of *gdhA*, removing 1311 bp of the *gdhA* ORF, was constructed in D39 using primer pairs gdhAKO-1/gdhAKO-2 (479 bp) and gdhAKO-3/gdhAKO-4 (498 bp).

Construction of capsule-less derivatives of D39 and its *glnA*, *glnR*, *glnRA* and *glnP* mutants was done as described (35), using primers PE21 and FI4. Mutants were checked by PCR and appearance. In addition, they adhere several orders of magnitude better than the encapsulated mutants.

Table 2. Oligonucleotide primers used in this study. [*]Stop-mutations are indicated in bold. Overlap of primers glnR-stop 1 and 2 is in italics. [#]6xHis-tag in bold. [$]Extra codon for lysine in italics. [**]Overlap with *emR* gene from pORI28 in bold.

Name	**Nucleotide sequence (5' to 3'); restriction enzyme sites underlined**	**Restriction site**
Ery-for	GCATGCATCGATTAGATCTC	-
Ery-rev	TAACGATTATGCCGATAACT	-
Spec_pORI38-Fp	CCCCAAGCTTCTAATCAAAATAGTGAGGAGG	*Hind*III
Spec_pORI38-Rp	CCCCAAGCTTACTAAACGAAATAAACGC	*Hind*III
R6_glnA-5	TGCTCTAGACTTTCTTCTATATTAGTATTAGTAAAGGTC	*Xba*I
R6_glnA-6	CGGAATTCGGTATTGCTGGATCAGGTATGCAC	*Eco*RI
R6_PglnP-1	CGGAATTCCGAAAGTCACTTGTTTTATCC	*Eco*RI
R6_PglnP-2	TGCTCTAGAAGGACATCTGAATGTTCCAGC	*Xba*I
R6_gdhA-4	CGGAATTCCGATGAAAACGGTATCGACTTCG	*Eco*RI
R6_gdhA-5	TGCTCTAGACTTAAACAATACCTTGTGCAATCATAGC	*Xba*I
R6_G6PDH-1	CGGAATTCGCTCTGGTAAGATTGATGG	*Eco*RI
R6_G6PDH-2	TGCTCTAGATTTTTATTCTAAGCGACCATC	*Xba*I
R6_arcA-3	TGCTCTAGACCGAGAAGACCTGAATTGGATGTG	*Xba*I
R6_arcA-4	CGGAATTCGGTAGGCTCAGGATTTTTCTCC	*Eco*RI
Pg6pdh-1	CGGAATTCGAAAGAGTTCTTAGATAAGG	*Eco*RI
Pg6pdh-2	TGCTCTAGACGAAAATTGTAACAATAACC	*Xba*I
glnP-OX1	CGAGCCATCATGAAGAAAAAATTTCTAGCATTTTTGC	*Rca*I
glnP-OX2	TGCTCTAGAGTAGAAAAACTGCAAGGAAATCC	*Xba*I
glnR_R6-9	GCCATCATGAAGGAAAAAGAATTTCGCC	*Rca*I
glnR-9-his[#$]	CGAGCCATCATG***AAA*****CATCATCATCATCATCAT**AAGGAAAA AGAATTTCGCCG	*Rca*I
glnR_R6-10	GCTCTAGAGTTTTCTCCTTAATCTATGAC	*Xba*I

glnA-his[#]	GGCGGTCTCTCATG**CATCATCATCATCATCAT**CCAATCACAG CTGCAGATATTCG	*Bsa*I
glnA_R6-8	GCTCTAGATGTGACTGTTTACCCACAG	*Xba*I
glnA_R6-1	GAATTCCATATGGGAAATAGTATCGGTGGAC	*Nde*I
glnA_R6-2	CGGCGACGTCGCAGCTGTGATTGGCATAAG	*Aat*II
glnA_R6-3	CATGCCATGGGGAAATTGATAATTATTTAGACC	*Nco*I
glnA_R6-4	GAAGATCTCTAGACGTATCTATATACC	*Bgl*II
glnR_R6-1	TGCTCTAGAGGTGGTGACTCAGCTGCCGC	*Xba*I
glnR_R6-2	CGGGATCCCCTTCATTTACAATTTCCTTC	*Bam*HI
glnR-stop 1[*]	*CGGTC**T**ACT**A**CATAACACTG*CCGATAGGAAAAACAGCC	-
glnR-stop 2[*]	*CAGTGTTATG**T**AGT**A**GACCG*ATCTATCGGCGCGTCAG	-
glnR-3	GAAGATCTACTTCAAGTGTTGGGTCCCC	*Bgl*II
glnPKO-1	AAAAAGTGCCAAGCCTAGAC	-
glnPKO-2[**]	**GAGATCTAATCGATGCATGC**TACCTAATGAGAAAATTGGG	-
glnPKO-3[**]	**AGTTATCGGCATAATCGTTA**CGAAACGCTTAGAAAAGAGG	-
glnPKO-4	TAATCATGGTCATGCCTTGC	-
gdhAKO-1	GATCGATTTGCCCTGTTCTTG	-
gdhAKO-2[**]	**GAGATCTAATCGATGCATGC**GCAGATGTCATATCGTTCTCC	-
gdhAKO-3[**]	**AGTTATCGGCATAATCGTTA**CTATGATTGCACAAGGTATTG	-
gdhAKO-4	AAGGAGTAGAGATGGCTATAG	-
G6PDH-4	TGCTCTAGACCAACACTTCAACCTCTTCCC	*Xba*I
G6PDH-5	CGGGATCCCCGAAAATTGTAACAATAACC	*Bam*HI
G6PDH-6	CATGCCATGGTCACCTATCGTCAAGATGGTCG	*Nco*I
G6PDH-7	GAAGATCTGGCCCACCGCTACAAACAAGC	*Bgl*II
PgdhA-2	TGCTCTAGATCTTTAGCAGATGTCATATC	*Xba*I
PgdhA-3	TGCTCTAGACATAAGACATTATAGCAG	*Xba*I
PgdhA-4	CGGAATTCGATATTTCCAAGAAAAACGTTCG	*Eco*RI
PE21	CTGGAACAACCATGACCTCCCTCG	-
FI4	CGCTGAACTTTTGTAGTTGCTTGGTCAAC	-
R6_glnP-1	CGGAATTCCATTTTTGAAGCTTGGAAGTC	Footprinting
R6_glnR-7	GCTCTAGACTGCCGATAGGAAAAACAGCC	Footprinting
R6_PglnR_FP	TTGTACGTGTTTGTGCGTG	Footprinting
R6_glnP-GFP1	CGGAATTCCATTTTTAGTCTCCTTTTCCG	Footprinting
PglnR-2	CGGAATTCCGATATTGATCGTATTCGTC	EMSA
PglnR-3	CGGAATTCATTATCAATTGACGTTTGTC	EMSA
PglnPQ-1	CGGAATTCGGCACTTTTTAATAGCAATTCAAG	EMSA
PglnPQ-2	TGCTCTAGAGGAATAAAATTAGCAAAAATGC	EMSA
PglnPQ-3	CGGAATTCGCGAAAAATATAACAATTTGCC	EMSA

Construction of lacZ fusions

Chromosomal transcriptional *lacZ* fusions were constructed with the integration plasmid pORI13 as described (23, 37). For *lacZ* fusions to *glnA*, *gdhA* and *zwf*, 600-to 800-bp fragments of the 3' ends of the genes were PCR amplified using primer pairs R6_glnA-5/R6_glnA-6, R6_gdhA-4/R6_gdhA-5 and R6_G6PDH-1/R6_G6PDH-2, respectively. These fragments were digested and cloned into the *Xba*I/*Eco*RI sites of PORI13, giving pTK8, pTK10, pTK11, respectively. The constructs were introduced into *S. pneumoniae* D39*nisRK*, and D39*nisRK* containing either the *glnA* (TK100) or *glnR* (TK105) mutation and clones were checked by PCR. Analogously, pTK9 and pTK12 were constructed with primers R6_PglnP-1/R6_PglnP-2 and R6_arcA-3/R6_arcA-4. These plasmids were used to generate chromosomal *lacZ* fusions to the *glnP* and *arcA* promoters.

The *glnQ-zwf* intergenic region was cloned into pORI13 using primers Pg6pdh-1/ Pg6pdh-2, giving pTK21.

The *lacZ* fusions to the *gdhA* promoter were constructed in pPP2 with primer pair PgdhA-2/PgdhA-4, giving a PCR product comprising the full-length promoter (P*gdhA*-1), and primer pair PgdhA-3/PgdhA-4, resulting in a PCR product without the predicted GlnR operator (P*gdhA*-2), using *E. coli* EC1000 as the cloning host. The constructs were introduced in *S. pneumoniae* strains D39 and TK102.

Construction of overexpression constructs

The *glnR*, *glnA* and *glnPQ* genes were PCR amplified with primer pairs glnR-9-his/glnR_R6-10, glnA-his/glnA_R6-8 and glnP-OX1/glnP-OX2, respectively, and cloned into the *Nco*I/*Xba*I sites of pNG8048E, giving pTK16, pTK15 and pTK17. In addition, the native *glnR* gene was cloned into pNG8048E using primers glnR-_R6-9/glnR_R6-10, giving pTK23.

Purification of GlnR and GlnA

Overexpression of N-terminally 6xHis-tagged GlnR and GlnA (H_6-GlnR and H_6-GlnA) was achieved with the nisin-inducible system (NICE) in strain *L. lactis* NZ9000 (27). Expression was induced with nisin in 1 L cultures at an OD_{600} of 0.6, using a 10^{-7} dilution (2 ng/ml) of nisaplin, which was prepared as described (23). After two hours of induction, cells were harvested and resuspended in 10 ml buffer A (0.25 M NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl, pH 8, 10% glycerol, 1 mM β-mercaptoethanol) with 1 mg/ml lysozyme and 1 tablet of protease inhibitor cocktail (Complete Mini, Roche). After 20 min of incubation on ice, cells were disrupted by shaking 5 times 1 min with 400 mg glass-beads (75-150 μm, Fischer

Scientific BV) per ml of cell suspension in a Biospec Mini-BeadBeater-8 (Biospec Products) and cell debris was removed by centrifugation. 1 ml Ni-NTA beads (Qiagen), pre-equilibrated in buffer A, was added to the cell lysate and protein binding was allowed for 1h at 4ºC, with continuous gentle shaking. Beads were washed 10 times with buffer A containing 20 mM imidazole after which H_6-GlnR and H_6-GlnA were eluted with buffer A containing 250 mM imidazole and subsequently with buffer A containing 350 mM imidazole. H_6-GlnA was dialysed against 2,000 times excess of buffer B (20 mM Tris-HCl, pH 8.5, 10% glycerol, 1 mM β-mercapto-ethanol) for 6 hrs at 4ºC. Since H_6-GlnR precipitated during dialysis, imidazole was removed by means of a PD-10 desalting column (Amersham Biosciences), using buffer C (20 mM Tris-HCl, pH 7.5, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercapto-ethanol), in which way precipation was not observed. Purified fractions contained >95% pure protein of the expected size with a concentration of between 0.2 and 1 mg protein/ml.

Electrophoretic mobility shift assays and DNAseI footprinting

EMSA's were performed essentially as described previously (10). PCR products of P*glnR* with and without the predicted GlnR operator were made with primer pairs glnR_R6-2/PglnR-2 (P*glnR*-1, 146 bp) and glnR_R6-2/PglnR-3 (P*glnR*-2, 84 bp), respectively. In the same way, PCR products spanning P*glnP* were generated with primer pairs PglnPQ-1/PglnPQ-2 (P*glnP*-1, 190 bp) and PglnPQ-2/PglnPQ-3 (P*glnP*-2, 131 bp), respectively. The binding buffer was composed of 20 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 1 mM dithiotreitol (DTT), 8.7% (w/v) glycerol, 62.5 mM KCl, 25 µg/ml bovine serum albumin, 50 µg/ml poly(dI-dC) and 3000-5000 cpm of [γ-^{32}P]ATP-labeled PCR product. Glutamine, glutamate, ammonium and purified H_6-GlnR and H_6-GlnA were added as specified in the Results section. Reactions (20 µl) were incubated for 20 min at 25ºC after which they were run on a 6% poly-acrylamide gel for 75 min at 90V.

DNAseI Footprinting was done essentially as described (10). 150.000 cpm of [γ-^{32}P]ATP-labeled PCR products of the *glnR* and *glnP* promoters, made with primer pairs R6_PglnR_FP/R6_glnR-7 (244 bp) and R6_glnP-1/R6_glnP-GFP1 (235 bp), respectively, were used as probes in 40 µl binding buffer (EMSA) containing 5 mM glutamine and purified H_6-GlnR and H_6-GlnA as specified in the Results section.

Enzyme assays

Cell-free extracts, used for the determination of glutamine synthetase (GlnA), biosynthetic glutamate dehydrogenase (GdhA) and glucose-6-phosphate dehydrogenase (Zwf) activity, were made from 1 or 2 ml of cells harvested in exponential phase of growth, which were resuspended in 250 µl 20 mM Tris (pH 7.5) and disrupted by shaking 1 min with 400 mg glassbeads (75-150 µm) in a Biospec Mini-BeadBeater-8. After removal of cell-debris by centrifugation, cell-free extracts were used in a concentration of 1/10 to 1/20 of the total volume of the assay mixture. The OD_{600} at which cells were harvested was used to calculate the enzyme activity per OD_{600} unit. Standard deviations were calculated from at least three independent replicate experiments. GlnA activity (transferase reaction) was determined as described (14). Biosynthetic glutamine synthetase activity of purified H_6-GlnA (ATP + L-glutamate => ADP + Pi + L-glutamine) was determined as described (12). Biosynthetic GdhA activity (2-oxoglutarate + NADPH + NH_4^+ => glutamate + $NADP^+$) was determined at 30ºC in a reaction mixture containing 50 mM Tris-HCl (pH 8.5), 35 mM 2-oxoglutarate, 80 mM NH_4Cl and 0.3 mM 2'-NADPH, by monitoring the decrease in absorption at 340 nm (A_{340}) caused by oxidation of NADPH. Catabolic GdhA activity was measured at 30ºC in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 70 mM L-glutamate, and 0.5 mM $NADP^+$, by monitoring the increase in A_{340}. Activity of Zwf (glucose-6-P + $NADP^+$ => gluconolactone-6-phosphate + NADPH) was measured in buffer containing 1 mM $NADP^+$, 2 mM glucose-6-phophate, 10 mM $MgCl_2$, 1 mM DTT and 20 mM Tris-HCl (pH 8.0), by monitoring the increase in A_{340} at 30ºC. Activity of β-galactosidase was determined as described (20), except that cells were permeabilized with a final concentration of 0.06 mg/ml CTAB (cetyltrimethyl ammonium bromide). During growth for β-galactosidase assays, no antibiotic selection was imposed.

Transcriptome analysis using *S. pneumoniae* DNA microarrays

DNA microarray experiments were performed essentially as described (46). RNA was isolated from 50 ml of cells grown to mid-exponential phase of growth (OD_{600} = 0.3) in GM17 (M17 containing 0.25% glucose) containing 0.5 mg/ml glutamine (GM17Gln). Cells were harvested by centrifugation for 1 min at 10,000 rpm at room temperature. Cell pellets were immediately frozen in liquid nitrogen and stored at -80°C. Pellets were resuspended in 500 µl 10 mM Tris-HCl, 1 mM EDTA ($T_{10}E_1$), pH 8.0, after which 50 µl 10% SDS, 500 µl

phenol/chloroform, 500 mg glass beads (75-150 µm), and 175 µl Macaloid suspension (Bentone MA) were added.

Synthesis of cDNA and indirect Cy-3/Cy-5-dCTPs labeling of 15-20 µg total RNA was performed with the CyScribe Post Labelling Kit (Amersham Biosciences) according to the supplier's instructions. Hybridization (16 h at 45°C) of labeled cDNA was performed in Ambion Slidehyb #1 hybridisation buffer (Ambion Europe) on super-amine glass slides (Array-It, SMMBC), containing technical replicates of amplicons representing 2087 ORFs of *S. pneumoniae* TIGR4 and 184 ORFs unique for *S. pneumoniae* R6. DNA microarrays were produced essentially as described (26, 46). Amplicon sequences are available at http://molgen.biol.rug.nl/publication/glnRAspn_data. Slides were scanned using a GeneTac LS IV confocal laser scanner (Genomics Solutions).

DNA microarray data analysis

Slide images were analysed using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD). Processing and normalization (LOWESS spotpin-based) of slides was done with the in-house developed *MicroPrep* software as described (46, 47). DNA microarray data were obtained from 3 independent biological replicates hybridised to three glass slides, of which one was a dye-swap. Expression ratios of mutant strain over the wild-type strain were calculated from the measurements of at least 5 spots. Differential expression tests were performed on expression ratios with a local copy of the Cyber-T implementation of a variant of the t-test. False discovery rates (FDRs) were calculated as described (46). A gene was considered differentially expressed when $p < 0.001$ and FDR < 0.05 and when at least 5 measurements were available.

Accession numbers

The DNA microarray data are available at http://molgen.biol.rug.nl/publication/glnRAspn_data. In addition, they have been deposited in the Gene Expression Omnibus GEO (www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE5088).

Reverse-transcriptase PCR

RNA isolation and cDNA synthesis was performed as described above, except that aminoallyl-dUTP was replaced by dTTP during cDNA synthesis. To confirm the absence of DNA contamination, reactions were also carried out without reverse transcriptase. 100 ng cDNA was used for each PCR reaction, and after 20 amplification cycles (30 sec, 95ºC; 30 sec, 52ºC; 60 sec, 72ºC) with primers G6PDH4 and G6PDH5 reactions were analysed on 1% agarose gels.

Adhesion assays

Adhesion of pneumococci to epithelial cells was studied essentially as desribed previously (21). Briefly, the human pharyngeal cell line Detroit 562 (ATCC CCL-138) was cultured in RPMI 1640 without phenol red (Invitrogen) containing 1 mM sodium pyruvate and 10% fetal calf serum (FCS). Aliquots of bacteria, grown to mid-exponential phase in GM17 and stored till use at -80°C, were thawed rapidly, harvested by centrifugation, and resuspended to $1x10^7$ CFU/ml in RPMI 1640 medium without phenol red containing 1% FCS. Monolayers of Detroit 562 in 24-well tissue culture plates were washed twice with 1 ml PBS, after which 1 ml of bacterial suspension was allowed to adhere for 2h at 37°C in a 5% CO_2 atmosphere. Subsequently, non-adherent bacteria were removed by three washes with 1 ml PBS, and the epithelial cells were detached by treatment with 200 µl of 25% Trypsin, 1 mM EDTA in PBS. Detroit 562 cells were lysed by the addition of 800 µl of ice-cold 0.025% Triton X-100 in PBS, and appropriate dilutions were plated on blood agar plates to count the number of adherent bacteria. This CFU count was first corrected mathematically to account for small differences in count in the initial inoculum, after which data were normalized so that the adhesion of the wild-type strain TK136 was expressed as 100%. Wild type and mutant pneumococci grew comparably in RMPI medium without Detroit 562 cells. All experiments were performed in triplicate and repeated at least three times. Significant differences between wild-type and mutants were calculated by the Mann Whitney t-test ($p<0.05$).

Results

Prediction of putative GlnR operators in *S. pneumoniae*

B. subtilis GlnR is known to repress genes that contain two copies of the inverted repeat 5'-TGTNAN$_7$TNACA-3' in their promoters (7, 8). This repeat is also present in the promoter regions of the *Lactobacillus rhamnosus* (48) and *Bacillus cereus* (33) *glnRA* operons. As the GlnR binding box seems so well conserved between species, we screened the entire genome of *S. peumoniae* R6 for the presence of putative GlnR operators using Genome2D (3).

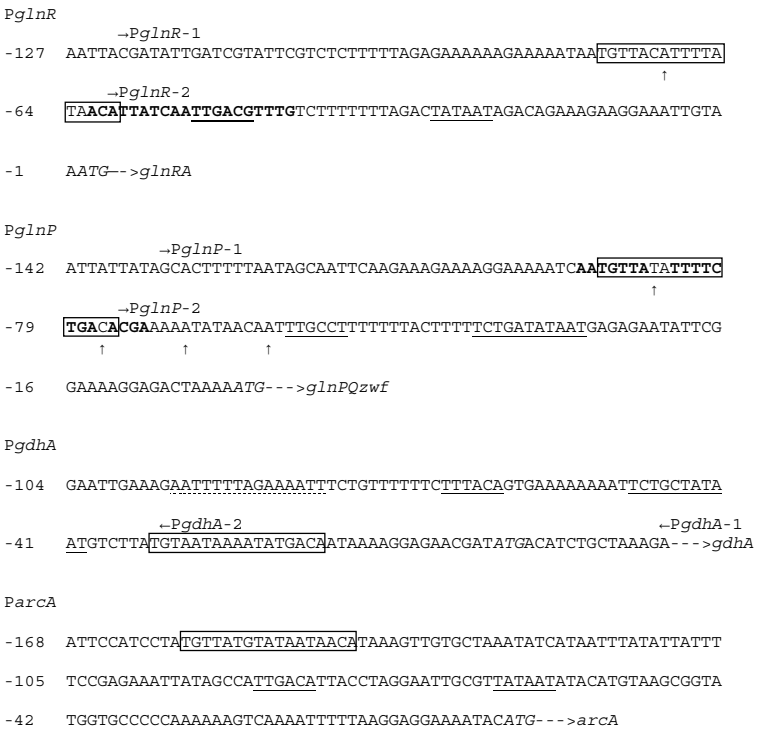

Figure. 1. Nucleotide sequences of the promoter regions of the indicated genes/operons of *S. pneumoniae* R6. Predicted -35 and (extended) -10 core promoter regions are underlined. Putative GlnR operators are boxed. Translational starts are in italics. Numbers indicate the base-positions relative to the translational start. A predicted CodY operator in the *gdhA* promoter is underlined with a dotted line. Bases in P*glnR* and P*glnP* that are in bold were protected in the DNAseI footprinting analyses (Fig. 6B) and vertical arrows below the sequences indicate hypersensitive bases. Horizontal arrows above P*glnR* (P*glnR*-1 and P*glnR*-2), P*glnP* (P*glnP*-1 and P*glnP*-2) and P*gdhA* (P*gdhA*-1 and P*gdhA*-2) indicate the locations of the primers used to make the promoter-truncations as used for Figures 4C and 6A.

Table 3. Summary of transcriptome comparison of *S. pneumoniae* strains D39 *glnR*-stop and D39 Δ*glnA* with D39 wild-type.

Gene name[a]	Function[b]	glnR-stop[c]	*glnA*[d]
SP0092	ABC transporter, substrate-binding protein	**1.5** (3.1e-7)	NDE[e]
SP0237	Ribosomal protein L17	**1.5** (2.0e-8)	NDE
SP0295	Ribosomal protein S9	**1.6** (3.6e-9)	**1.6** (4.3e-5)
SP454	Hypothetical protein	**1.5** (2.2e-4)	NDE
glnR (SP0501)	Nitrogen regulatory protein	**5.0** (0.0)	**1.7** (3.8e-4)
glnA (SP0502)	Glutamine synthetase	**5.1** (0.0)	**-2.4** (5.3e-5)
SP0922	Carbon-nitrogen hydrolase family protein	NDE	**1.6** (2.0e-5)
SP0964	Dihydroorotate dehydrogenase	NDE	**1.8** (7.3e-6)
SP0965	Endo-beta-N-acetylglucosaminidase	**-1.6** (2.4e-8)	NDE
SP1111	Hypothetical protein	NDE	**-3.7** (5.0e-4)
glnP(SP1241)	Glutamine ABC transport and substrate binding prot.	**4.3** (2.8e-15)	**2.8** (3.6e-12)
glnQ (SP1242)	Glutamine ABC transport and substrate binding prot.	**4.0** (0.0)	**2.5** (8.4e-11)
zwf (SP1243)	Glucose-6-phosphate dehydrogenase	**2.8** (4.7e-15)	**1.6** (7.3e-7)
SP1275	Carbamoyl-phosphate synthase large subunit	**2.4** (1.6e-5)	**1.5** (7.9e-7)
SP1276	Carbamoyl-phosphate synthase small chain	NDE	**1.6** (1.4e-8)
SP1277	Aspartate carbamoyltransferase	**1.8** (1.1e-4)	**1.8** (5.0e-7)
gdhA (SP1306)	NADPH-dependent biosynthetic glutamate dehydrogenase	**2.2** (6.9e-12)	**2.0** (1.0e-9)
SP1354	Ribosomal protein L7/L12	**1.8** (3.1e-9)	NDE
SP1936	Type II restriction-modification system regulatory protein	**1.6** (9.3e-6)	NDE
SP2055	Alcohol dehydrogenase, zinc-containing	**1.5** (2.2e-6)	NDE
SP2060	Pyrrolidone-carboxylate peptidase	NDE	**-1.7** (2.8e-5)
SP2063	LysM domain protein, authentic frameshift	**-1.9** (1.1e-11)	**-2.3** (6.5e-9)
arcA (SP2148)	Arginine deiminase	**1.6** (4.4e-4)	NDE

[a]Gene numbers refer to TIGR4 locus tags.

[b]TIGR annotation.

[c]Ratio's > 1.5 or < -1.5 (D39 *glnR*-stop compared to D39) in bold, p-values in parenthesis.

[d]Ratio's > 1.5 or < -1.5 (D39 Δ*glnA* compared to D39) in bold, *p*-values in parentheses.

[e]NDE, not significantly differentially expressed.

Predicted operators with the highest similarity to the *B. subtilis* consensus sequence were present in the promoter regions of *glnR* (*spr0443*); *glnP* (*spr1120*), encoding a

glutamine ABC transporter substrate binding protein; *gdhA* (*spr1181*) encoding a NADP(H)-specific glutamate dehydrogenase; and *arcA* (*spr1955, spr1956*), encoding arginine deiminase (Fig. 1). Re-searching the R6 genome with a weight matrix built from these putative operators did not reveal additional putative GlnR operators.

The regulon of GlnR and GlnA in *S. pneumoniae*

To investigate the role of GlnR in *S. pneumoniae* in more detail, we constructed a *glnR* mutant in strain D39. To preserve the *glnRA* operon structure, two consecutive stop-codons were introduced in the beginning of the *glnR* open reading frame (ORF), specifying amino acids in the middle of the predicted helix-turn-helix DNA-binding motif.

DNA microarray analyses were performed of *S. pneumoniae* D39 wild-type and its isogenic *glnR* mutant grown in the nitrogen-rich medium GM17, supplemented with 0.5 mg/ml glutamine (GM17Gln). This amino acid is assumed to be a co-repressor of GlnR in *B. subtilis* (38), and we expected it to also induce repression of GlnR targets in *S. pneumoniae*. The operons/genes that were most highly up-regulated in the *glnR* mutant were *glnRA*, *glnPQ*, and *gdhA* (Table 3), all of which have a GlnR operator in their promoter regions (Fig. 1). The *arcA* gene, which also contains a putative GlnR operator in its promoter, was only weakly up-regulated. Remarkably, also *zwf*, encoding the key enzyme glucose-6-phosphate dehydrogenase of the pentose phosphate pathway, was up-regulated. This gene lies downstream of and in the same orientation as *glnPQ*.

To investigate the influence of GlnA on the expression of the identified GlnR targets, a comparison of the transcriptomes of wild-type D39 with its isogenic *glnA* mutant, grown in GM17Gln, was performed. This showed that *glnR*, *glnP*, *glnQ* and *gdhA* were, like in the *glnR* mutant, also up-regulated in the *glnA* mutant (Table 3), indicating that GlnA is necessary for the functioning of GlnR.

Besides the genes mentioned above, *SP2063*, encoding a predicted LysM domain containing protein, not directly involved in glutamine metabolism, was 2-fold down-regulated in both the *glnR* and the *glnA* mutant. Interestingly, two degenerate GlnR boxes (5'-TGTGACAGAGACCTAACA-3' and 5'-TGTTATTAGCGTCAACA-3') are present in the promoter region of this gene.

In both the *glnR* and *glnA* mutant, genes predicted to encode proteins involved in pyrimidine metabolism (*SP1275, SP1276, SP1277*, *SP0954*) were moderately up-regulated, which seems logical as glutamine is a precursor of pyrimidine. However, as no GlnR operator could be identified upstream of any of these genes, the up-regulation is likely to be an indirect

effect caused by altered intracellular glutamine/glutamate levels. Furthermore, a number of other genes of various functions were moderately up-regulated in either the *glnR* or the *glnA* mutant.

Chromosomal transcriptional *lacZ* fusions were used to confirm that in both the *glnR* and the *glnA* mutant, expression of *glnA*, *glnP*, *zwf* and *gdhA* was de-repressed (Figures 2A, 3A, 3B, and 4A). In addition, enzymatic activity assays showed that the observed effects on transcription corresponded with altered activities of GlnA, GdhA and Zwf in the *glnR* and *glnA* mutants (Figures 2B, 3C, and 4B). No strong effect of the *glnR* or the *glnA* mutation on the expression of a chromosomal P*arcA-lacZ* transcriptional fusion was observed in a range of different media (data not shown).

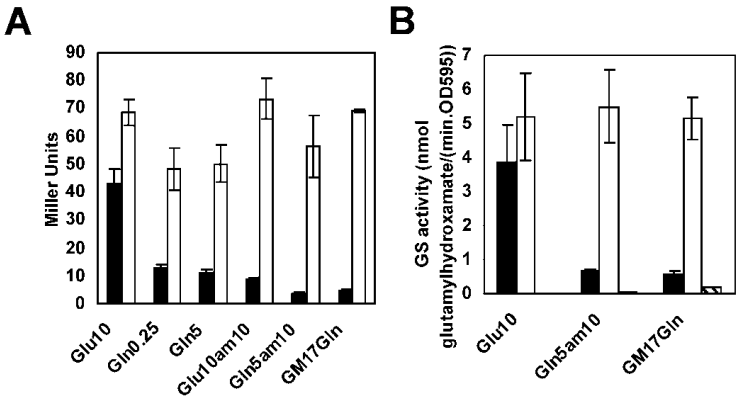

Figure. 2. Expression of *glnA* in *S. pneumoniae* D39 (black bars) and its *glnR* (white bars) and *glnA* (diagonally hatched bars) mutants in various media. **(A)** β-galactosidase activity in strains TK110 (D39*nisRK glnA-lacZ*) and TK120 (D39*nisRK glnR*-stop *glnA-lacZ*) in CDM supplemented with 10 mM glutamate (glu10), 0.25 mM glutamine (Gln0.25), 5 mM glutamine (Gln5), 10 mM glutamate and 10 mM NH_4Cl (Glu10am10), 5 mM glutamine and 10 mM NH_4Cl (Gln5am10), and in GM17 with 0.5 mM glutamine (GM17Gln), which is indicated at the x-axis. (**B)** GlnA activity in strains D39, TK102 (D39 *glnR*-stop) and TK103 (D39 Δ*glnA*) in the media indicated on the x-axis.

Nitrogen source-dependent regulation of *glnRA* and *glnPQ* by GlnR

To address the role of GlnA substrates in the observed GlnR-dependent regulation in *S. pneumoniae*, expression of the chromosomal *glnA-lacZ* and *glnP-lacZ* fusions was measured in a chemically defined medium (CDM), to which glutamine, glutamate and ammonium were added in varying amounts (Fig. 2A). In CDM with only glutamate, *glnA* expression was similar in the wild-type and the *glnR* mutant. In contrast, glutamine led to

repression of *glnA* expression in the wild-type already at a relatively low concentration (0.25 mM). A higher glutamine concentration did not lead to stronger repression in the wild-type. However, when besides glutamine ammonium was included, *glnA* expression could be further repressed. The combination of glutamate and ammonium also gave rise to repression of *glnA* expression in the wild-type. None of the above combinations caused repression of *glnA* expression in the *glnR* mutant. GlnA enzymatic activity is regulated in the same way (Fig. 2B).

Regulation of *glnP* in response to glutamate, glutamine and ammonium is very similar to that of *glnA* (Fig. 3A). Derepression of *glnP* expression is also seen in the *glnA* mutant, albeit to a somewhat lower extent than in the *glnR* mutant. This could indicate that, in the absence of GlnA, GlnR is still able to exert a weak repressive effect on the expression of *glnP*.

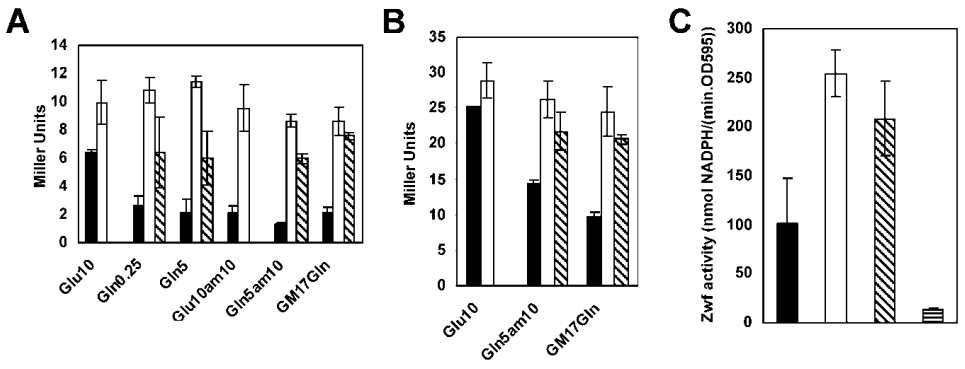

Figure 3. Expression of *glnPQ* and *zwf* in *S. pneumoniae* D39 (black bars) and *glnR* (white bars) and *glnA* (diagonally hatched bars) mutants in various media. **(A)** Beta-galactosidase activity in strains TK111 (D39*nisRK* P*glnP-lacZ*), TK121 (D39*nisRK glnR*-stop P*glnP-lacZ*) and TK127 (D39*nisRK* Δ*glnA* P*glnP-lacZ*) in the media as indicated on the x-axis (see Fig. 2 for abbreviations). **(B)** Beta-galactosidase activity in strains TK112 (D39*nisRK zwf-lacZ*), TK123 (D39*nisRK glnR*-stop *zwf-lacZ*) and TK130 (D39*nisRK* Δ*glnA zwf-lacZ*) in the media indicated on the x-axis. **(C)** Zwf activity in D39, TK102 (D39 *glnR*-stop), TK103 (D39 Δ*glnA*) and TK107 (D39 Δ*zwf*, horizontally hatched bars) in GM17Gln.

Regulation of *zwf* by GlnR and GlnA occurs via the *glnP* promoter

The DNA microarray results showed that *zwf*, which lies downstream of *glnPQ*, is also up-regulated in the *glnR* and *glnA* mutants. RT-PCR demonstrated that *zwf* lies on the same transcript as *glnPQ* and thus forms an operon with these genes (data not shown). To

examine whether *zwf* is only transcribed from P*glnP*, or also from a possible promoter in the *glnQ-zwf* intergenic region, the latter was cloned upstream of *lacZ* in pORI13 and introduced in the RepA$^+$ strain D39*repA* (23) and its *glnR* mutant. Promoter activity was present in this fragment (~5 Miller units), which was not dependent on GlnR (data not shown). Regulation of *zwf* by GlnR and GlnA was similar to, but weaker than regulation of *glnPQ* (Fig. 3B and C), which can be explained by the presence of the second promoter upstream of *zwf*. Thus, expression of *zwf* initiates from two promoters, a GlnR-dependent promoter upstream of *glnP* and a second promoter in the *glnQ-zwf* intergenic region.

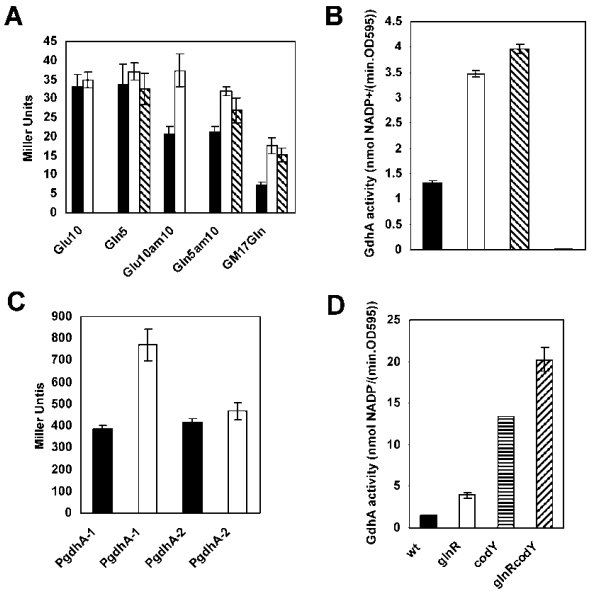

Figure 4. Expression of *gdhA* in *S. pneumoniae* D39 and *glnR*, *glnA* and *codY* mutant derivatives. **(A)** β-galactosidase activity in strains TK111 (D39*nisRK gdhA-lacZ*; black bars), TK121 (D39*nisRK glnR*-stop *gdhA-lacZ*, white bars) and TK127 (D39*nisRK* Δ*glnA gdhA-lacZ*, hatched bars) in the media indicated on the x-axis (see Fig. 2 for abbreviations). **(B)** GdhA biosynthetic activity in D39 (black bar), TK102 (D39 *glnR*-stop, white bar), TK104 (D39 Δ*glnA* hatched bar) and TK strains in GM17Gln. **(C)** Beta-galactosidase activity in strains TK132 (D39 Δ*bgaA*::P*gdhA*-1-*lacZ*), TK133 (D39 Δ*bgaA* P*gdhA*-2-*lacZ*), TK134 (D39 *glnR*-stop Δ*bgaA*::P*gdhA*-1-*lacZ*) and TK135 (D39 *glnR*-stop Δ*bgaA*::P*gdhA*-2-*lacZ*) grown in GM17Gln. Black bars represent D39 background and white bars the *glnR* mutant background. PgdhA-1, full-length *gdhA* promoter. PgdhA-2, *gdhA* promoter without the predicted GlnR operator. **(D)** GdhA activity in D39, TK102 (D39 *glnR*-stop), WH101 (D39 Δ*codY*) and TK108 (D39 *glnR*-stop Δ*codY*) mutant strains grown in GM17Gln.

Regulation of *gdhA* by GlnR and CodY

Despite the fully conserved GlnR operator in the *gdhA* promoter, regulation of *gdhA* by GlnR and GlnA in GM17Gln and CDM was weaker than regulation of *glnPQ* and *glnRA* (Fig. 4A and B). However, expression of an ectopic *lacZ* fusion to the full-length *gdhA* promoter (P*gdhA*-1) and to a truncated version without the GlnR box (P*gdhA*-2, see also Fig. 1) showed that deletion of the predicted GlnR operator abolished the GlnR-dependent repression of P*gdhA* (Fig. 4C), demonstrating that the predicted GlnR operator in the *gdhA* promoter is functional.

Interestingly, in the *S. pneumoniae* R6 genome putative CodY operator sequences are present in the promoter regions of, amongst others, *gdhA* and *zwf* (11). To examine whether CodY regulates these genes in *S. pneumoniae*, the activity of the corresponding enzymes was measured in a *codY* deletion mutant. No effect of the *codY* deletion was seen on the activity of Zwf in GM17Gln (data not shown), but activity of GdhA was strongly increased in the *codY* mutant (Fig. 4D). In a *glnRcodY* double mutant, GdhA activity was even higher than in the *codY* mutant, indicating that GlnR and CodY independently repress *gdhA* in *S. pneumoniae* (Fig. 4D).

glnPQ* encodes the main glutamine/glutamate transport operon in *S. pneumoniae

Of the genes encoding predicted glutamine transporters in the R6 genome (19), *glnPQ* were the only ones found to be regulated by GlnR. To investigate the role of *glnPQ* in glutamine metabolism, a deletion of *glnP*, encoding the permease component of the GlnPQ ABC-transporter, was constructed in D39. Whereas *S. pneumoniae* D39 is able to grow in CDM containing glutamine (Fig. 5A) or glutamate (23), but not in their absence, the *glnP* mutant was not able to grow in CDM with either glutamine (Fig. 5A) or glutamate (data not shown). This phenotype could be complemented by *in trans* expression of *glnPQ* from a nisin-inducible promoter (Fig. 5A). Moreover, addition of the dipeptide Gly-Gln to the CDM also rescued growth of the *glnP* mutant (Fig. 5A), while this was not the case with the dipeptide Phe-Gly (data not shown). These data indicate that *glnPQ* encode the only actively expressed glutamine and glutamate uptake system in *S. pneumoniae* under these conditions.

GlnA activity was increased in the *glnP* mutant in GM17 (Fig. 5B), although to a lower extent as in the *glnR* mutant (Fig. 2B). To investigate whether regulation of *glnA* is affected in the *glnP* mutant, the effect of casitone as the nitrogen source in the medium was tested. Casitone, an enzymatic (pancreatic) digest of casein, consists of casein-derived peptides and contains no free glutamine and only a very low level of free glutamate

(http://www.bd.com/ds/technicalCenter/misc/bionutrientmanual.pdf). Growth of the *glnP* mutant in CDM containing 2% casitone as the only nitrogen source was the same as that of the wild-type strain (data not shown), indicating that the uptake of peptides can by-pass the inability to take up glutamine and glutamate. The addition of casitone, like glutamine, to GM17 resulted in an approximately 2-fold reduced GlnA activity in the wild-type strain, but not in the *glnP* mutant (Fig. 5B). Thus, besides GlnA, also GlnPQ appear to be necessary for efficient repression by GlnR.

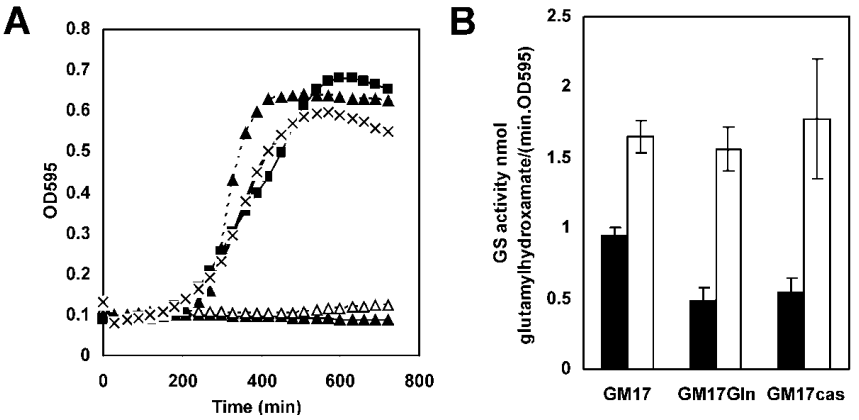

Figure 5. GlnPQ is the main glutamine/glutamate ABC transporter in *S. pneumoniae* D39. **(A)** Growth of D39 (squares), TK106 (D39 Δ*glnP*, white triangles) and TK106 containing plasmid pTK17 (dotted line, nisin (2 ng/ml) induced *glnPQ* expression; solid line, without nisin) in CDM with 1 mM glutamine. Crossed dotted line is growth of TK106 in CDM with 10 mM of the dipeptide Gly-Gln. **(B)** GlnA activity in strains D39 (black bars) and TK106 (white bars), grown in GM17, GM17Gln and GM17 with 2% casitone (GM17cas).

Binding of GlnR to P*glnP* and P*glnR* is GlnA-dependent

The transcriptional data presented above show that the activity of GlnR is dependent on GlnA. Therefore, we investigated whether GlnA is required for the binding of GlnR to the *glnR* and *glnP* promoters *in vitro*. For this we used a His-tagged variant of each protein (H_6-GlnA and H_6-GlnR). Nisin-induced expression of H_6-GlnA in strain TK100 restored growth in CDM with glutamate and no glutamine (data not shown). Nisin-induced expression of H_6-GlnR in the *glnR* mutant (TK105) led to 5-fold lower GlnA activity in CDM with 5 mM glutamine and 10 mM ammonium (data not shown). In CDM with 10 mM glutamate, the level of repression was still 4-fold, although the effect was weaker at low nisin-concentration. With wild-type GlnR, the repressive effect was also 5-fold in CDM with 5 mM glutamine and 10 mM ammonium, but only 2-fold in CDM with 10 mM glutamate. These data indicate that

H_6-GlnA and H_6-GlnR are functional, although the latter seems to respond in a less sensitive way to its assumed co-repressor glutamine.

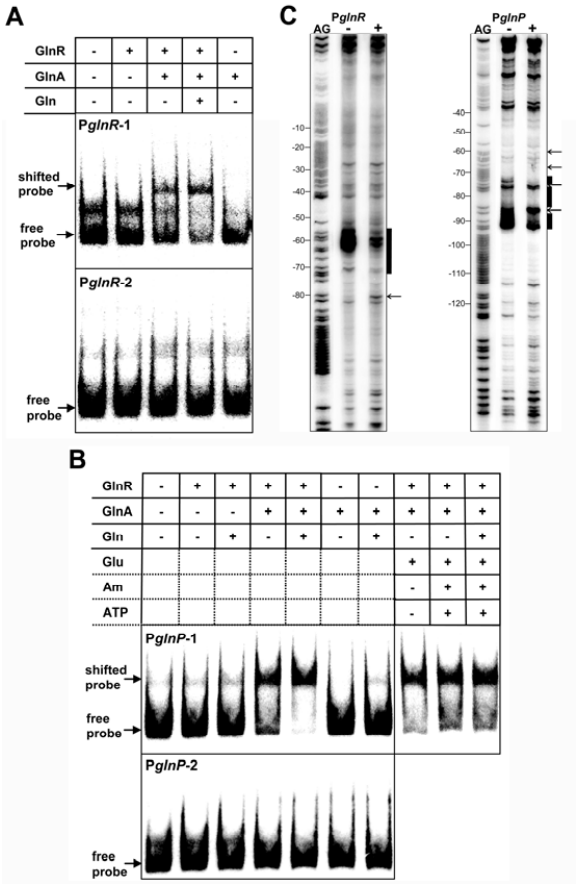

Figure 6. *In vitro* interaction of GlnR with the *glnR* and *glnP* promoters. **(A) (B)**, EMSA of binding of GlnR to the *glnR* (P*glnR*-1) and *glnP* (P*glnP*-1) promoter regions and to truncated *glnR* (P*glnR*-2) and *glnP* (P*glnP*-2) promoters, lacking their respective GlnR operators. H_6-GlnR, H_6-GlnA were added as indicated above the panels in concentrations of 400 nM (monomer*) and 1.5 µM (monomer*), respectively and glutamine (Gln), glutamate (Glu), ammonium (Am) and ATP were all added at 5 mM. The higher band seen for the free P*glnR* probe is probably single stranded DNA (10). **(C)** DNAseI footprint of P*glnR* and P*glnP* in the absence (-) and presence of 400 nM H_6-GlnR monomer* and 1.5 µM H_6-GlnA monomer*. Glutamine was present in all reactions in a concentration of 5 mM. Protected regions are indicated by black bars. Arrows indicate hypersensitive sites. Numbers on the left indicate bp positions relative to the translational starts of *glnP* and *glnR*. AG, Maxam-Gilbert sequence ladder. *GlnR is likely active as a dimer (7, 13, 17) and GlnA as a dodecamer (1).

H_6-GlnR alone at the concentration shown did not bind to P*glnR* and P*glnP* (Fig. 6A and B), also not in the presence of glutamine. However, in the presence of H_6-GlnA, binding of H_6-GlnR to P*glnR* and P*glnP* was observed (Fig. 6A and B). At a 5-10 times higher H_6-GlnR concentration (2 to 4 μM), a shifted band at the same position could be observed in the absence of H_6-GlnA (data not shown), which corresponds with the transcriptional data, showing some weak repression by GlnR in the *glnA* mutant (Table 3, Fig. 3A). No binding of H_6-GlnR was seen with the controls, the promoters without their GlnR operators (Fig. 6A and B).

DNAse I footprinting showed that, in the presence of H_6-GlnA, H_6-GlnR specifically reduces DNAseI sensitivity of the predicted GlnR operator in P*glnP* (Fig. 6C and Fig. 1). Remarkably, the protected region in P*glnR* only partially overlapped with the predicted GlnR operator (Fig. 6B and Fig. 1), suggesting that GlnR binds in a different manner to this promoter than to P*glnP*.

In contrast to what would be expected from the expression data, GlnA-dependent binding of GlnR to P*glnP* and P*glnR* was only weakly stimulated by the addition of glutamine (Fig. 6A and B). This could be explained by the observation mentioned above, that H_6-GlnR seems to be less sensitive to glutamine than the native protein. Addition of the other GlnA substrates glutamate, ammonium, ATP and AMP alone or in combination did not alter the observed GlnA-dependency of the GlnR-DNA interaction at P*glnP* (Fig. 6A). Thus, although GlnA is required for the binding of GlnR to the GlnR operators in the *glnP* and *glnR* promoters, this effect was not modulated by GlnA substrates.

GlnA and GlnP contribute to adhesion to pharyngeal epithelial cells

The crucial first step of pneumococcal virulence is the colonization of the nasopharynx. Therefore, we tested the ability of *glnR*, *glnA*, *glnRA* and *glnP* mutants to adhere to the human pharyngeal epithelial cell line Detroit 562. These mutants were created in the capsule-less background strain D39 Δ*cps*, as unencapsulated strains tend to show higher levels of adhesion ((34), data not shown). The *glnP*, *glnA* and *glnRA* mutants displayed a significantly decreased adhesion to the pharyngeal epithelial cells compared to the capsule-less wild-type strain (Fig. 7). Thus, both GlnP and GlnA could play a role in colonization of the nasopharynx. However, since most pneumococcal isolates are encapsulated, the actual contribution of these to proteins to virulence remains uncertain.

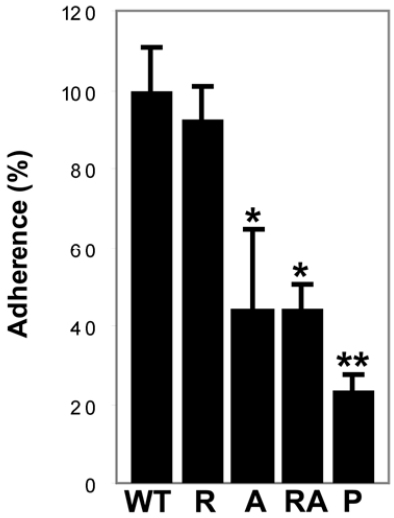

Figure 7. Adhesion of *S. pneumoniae* strains TK136 (D39 Δ*cps*, WT), TK137 (D39 *glnR*-stop Δ*cps*, R), TK138 (D39 Δ*glnA* Δ*cps*, A), TK139 (D39 Δ*glnRA* Δ*cps*, RA) and TK140 (D39 Δ*glnP* Δ*cps*, P) to Detroit 562 human pharyngeal epithelial cells. Adhesion is given in % relative to TK136. *p=0.0011, **p<0.0001.

Discussion

In this study, we characterized the regulation of glutamine and glutamate metabolism mediated by GlnR and GlnA in the human pathogen *S. pneumoniae*. Previously, GlnR-dependent regulation of nitrogen metabolism has been thoroughly studied in *B. subtilis*. The only target that *B. subtilis* GlnR shares with GlnR from *S. pneumoniae* is *glnRA* (8). In addition, *B. subtilis* GlnR is a repressor of the *ureABC* operon (13, 49) and *tnrA* (13), which are absent in *S. pneumoniae* (19). Another difference is that while GlnR is a repressor of *glnPQ* and *gdhA* in *S. pneumoniae*, in *B. subtilis* the catabolic glutamate dehydrogenase gene *rocG* is regulated by CcpA, RocR and AhrC (4), and the *glnQH* glutamine transport operon is activated by TnrA (53).

We found that also expression of *zwf*, encoding a putative glucose-6-phosphate dehydrogenase, is regulated by GlnR. This enzyme catalyzes the first reaction in the pentose phosphate pathway, which provides the cell with NADPH and ribose-5-phosphate, a building block of nucleic acids. As glutamine is a precursor for the synthesis of nucleotides as well, it might be advantageous for *S. pneumoniae* to coordinate *zwf* expression with glutamine metabolism.

Our data suggest that the regulation by *S. pneumoniae* GlnR depends on a conserved inverted repeat. The *B. subtilis* GlnR targets contain 2 copies of the same inverted repeat in their promoter regions (7, 8). *S. pneumoniae* GlnR resembles *B. subtilis* TnrA in this respect, as TnrA activates or represses promoters containing only one copy of this repeat (53).

The distance between the GlnR operator and the -35 box in P*glnR* is 7 bp, for P*glnP* it is 16 bp. GlnR boxes are also present at a distance of 5 to 7 bp from the -35 in the *glnR* promoters of the *S. pneumoniae* relatives *S. pyogenes*, *S. agalactiae* and *S. mutans*. Moreover, GlnR operators are located in the *glnP* promoters of *S. pyogenes* and *S. agalactiae*, which, like in *S. pneumoniae*, have a 16-bp spacing with the -35 sequence. As the spacing in the *glnP* promoters is 9 to 10 bp longer than in the respective *glnR* promoters, regulation by GlnR via these operators might be helix-side dependent in these organisms.

The GlnR operator in P*gdhA* confers a less pronounced GlnR-dependent effect than the GlnR operators in P*glnP* and P*glnR*, although the inverted repeat is perfectly conserved. The same accounts for the GlnR operator in P*arcA*. In P*glnR* and P*glnP* there is a stretch of A's immediately upstream of the repeat and a stretch of T's between the two half-sites. These stretches might explain the more efficient transcriptional repression of P*glnP* and P*glnR* than of P*arcA* and P*gdhA*, as AT-rich stretches on these positions of *B. subtilis* P*nrgAB* enhance TnrA-dependent transcriptional activation (51).

Both GlnR and CodY function as a repressor of *gdhA* in *S. pneumoniae*, of which CodY seems to be the more important regulator. Furthermore, both regulators control *gdhA* transcription independently of each other, which is in agreement with the location of their operators, that for CodY lying upstream of the -35 and the GlnR operator downstream of the -10 in P*gdhA*. In *B. subtilis* CodY controls the cellular nutritional and energy status (20, 21). Although GdhA is obviously connected to glutamine metabolism, the observation that *gdhA* expression is, next to GlnR, also regulated by CodY in *S. pneumoniae*, might indicate that GdhA is an important control point of the cellular nutritional status in this bacterium.

We show that GlnR DNA binding is dependent on GlnA, in contrast to the situation in *B. subtilis*, where GlnR alone binds with high affinity to its target promoters in the absence of any effectors (8). Since a high concentration of GlnR alone led to a shifted band at the same position as in the presence of GlnA, it is unlikely that GlnR and GlnA bind as a complex to the DNA. It might be that GlnA induces a conformational change or multimerization of GlnR, which increases its DNA binding affinity. Next to GlnA, also GlnP seems important for activity of *S. pneumoniae* GlnR. Although GlnR and GlnA alone were sufficient for *in vitro* binding to the *glnP* and *glnR* promoters, it could be that *in vivo* both GlnPQ and GlnA are needed for optimal activity of GlnR.

Our results and previous STM screens (18, 28, 36) implicate a role for both GlnP and GlnA in pneumococcal adhesion to human pharyngeal cells, which is a prerequisite to invade the host. Previously, GlnQ was shown to be required for adhesion of *S. pyogenes* to fibronectin and epithelial cells of the respiratory tract (43). However, it remains to be investigated whether the effect of GlnPQ on adhesion by *S. pneumoniae* and *S. pyogenes* is caused by a general effect of distorted glutamine metabolism on e.g. the cell-surface composition or that GlnPQ are directly involved. We are currently analysing *glnR*, *glnA*, *glnP*, and *gdhA* mutant strains in several *in vivo* mouse models, to get more insight in the role of glutamate and glutamine metabolism during infection by *S. pneumoniae*.

Acknowledgements

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CHAPTER 6

Site-specific contributions of glutamine-dependent regulator GlnR and GlnR-regulated genes to virulence of *Streptococcus pneumoniae*

GlnR-regulon and pneumococcal virulence

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Abstract

The transcriptional regulator GlnR of *Streptococcus pneumoniae* is involved in the regulation of glutamine and glutamate metabolism, controlling the expression of the *glnRA* and *glnPQ-zwf* operons as well as the *gdhA* gene. To assess the contribution of the GlnR-regulon to virulence, D39 wild-type and mutants lacking genes of this regulon were tested in an *in vitro* adherence assay and murine infection models. All mutants, except Δ*glnR,* were attenuated in adherence to human pharyngeal epithelial Detroit 562 cells, suggesting contribution of these genes to adherence during colonization of humans. During murine colonization only the Δ*glnA* and *glnP-glnA* double mutant (Δ*glnAP*) were attenuated, in contrast to *ΔglnP*, indicating that the effect is caused by the lack of GlnA expression. In our pneumonia model, only Δ*glnP* and Δ*glnAP* showed a significantly reduced number of bacteria in the lungs and in blood, indicating that GlnP is required for survival in the lungs and possibly for dissemination to the blood. In intravenously infected mice, *glnP* and *glnA* are individually dispensable for survival in the blood, whereas Δ*glnAP* was avirulent. Finally, transcriptome analysis of the Δ*glnAP* showed that many genes involved in amino acid metabolism were upregulated. This signifies the importance of glutamine / glutamate uptake and synthesis for full bacterial fitness and virulence. In conclusion, several genes of the GlnR-regulon are required at different sites during pathogenesis, with *glnA* contributing to colonization and survival in the blood and *glnP* important for survival in the lungs and, possibly, efficient transition from the lungs to the blood.

Introduction

Streptococcus pneumoniae is often carried asymptomatically in the human nasopharynx. However, this Gram-positive bacterium can cause disease upon dissemination to other sites of the body, such as otitis media, pneumonia, septicemia, and meningitis (3). To survive at various sites in the host *S. pneumoniae* needs to metabolize available nutrients. In line with this, several systems involved in nutrition and metabolism have been suggested to be important for virulence. For example, PsaA is part of a Mn(II) uptake system and contributes to colonization and invasive disease (2, 13), while the Ami-AliA/AliB oligopeptide uptake system contributes to colonization only (15).

Nitrogen metabolism is of utmost importance for bacterial survival, and is therefore strictly regulated. Glutamine and glutamate serve as main sources of nitrogen in the cell. We have recently shown that in pneumococcus, expression of genes involved in glutamine metabolism is regulated by the transcriptional regulator GlnR, which has a similar function in *Bacillus subtilis* and *Lactococcus lactis* (5, 17, 19) (Fig.1). In the pneumococcus, the GlnR-regulon consists of two operons, *glnRA* and *glnPQ-zwf*, and the *gdhA* gene. The gene *glnA* encodes glutamine synthetase GlnA, which forms glutamine out of glutamate and ammonium while hydrolyzing ATP. The *glnPQ* genes encode the main glutamine / glutamate transporter, and *zwf* encodes glucose-6-phosphate 1-dehydrogenase, an enzyme involved in pentose metabolism (17). The gene *gdhA* encodes glutamate dehydrogenase, which converts 2-oxoglutarate and ammonium to glutamate, thereby hydrolyzing NAD(P)H. We have demonstrated that GlnR binds to a conserved operator sequence present in the promoter regions of its target genes (17). Regulation by GlnR is dependent on GlnA, as the GlnR targets are derepressed in a *glnA* mutant and GlnA stimulates binding of GlnR to its operator sequence (17). In addition, GlnR-regulation is responsive to glutamine and ammonium (17, 19).

Importantly, expression of *gdhA* has been shown to be under control of a second regulatory protein, CodY (9, 17). This transcriptional repressor has been studied extensively in *B. subtilis* and *L. lactis* (6, 23, 28, 30), and recently in pneumococcus (9). Targets of CodY have been shown to consist mainly of genes involved in amino acid biosynthesis and uptake. It can be expected that GdhA plays an important role in central amino acid metabolism as the expression of its gene is tightly regulated by GlnR and CodY. In *B. subtilis*, a GlnR-ortholog is present, TnrA, which regulates various genes, including genes involved in nitrogen metabolism (30, 33). TnrA activates several genes during low nitrogen availability and its DNA recognition site is similar to that of GlnR (5, 30, 33). In pneumococcus no TnrA

homologue is present, indicating differences in regulation of nitrogen metabolism with *B. subtilis*.

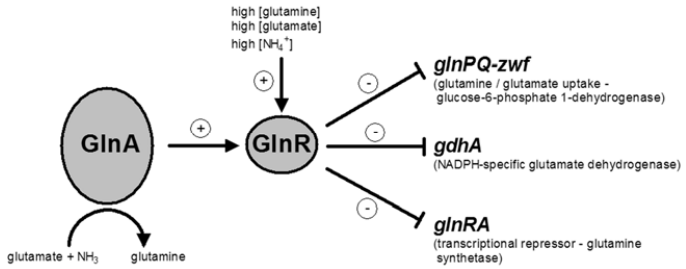

Figure 1. The pneumococcal GlnR-regulon. GlnR regulates the expression of the *glnRA* and *glnPQ-zwf* operons, and the *gdhA* gene. Repression of gene expression by GlnR is responsive to high concentrations glutamine, glutamate, and ammonium. Moreover, regulation by GlnR requires cellular GlnA for full repression of target genes.

Signature-tagged mutagenesis screens have suggested that several pneumococcal genes involved in glutamine metabolism are required for full virulence (8, 20, 27). In group B streptococci, a mutant deficient in *glnQ*, the gene encoding a glutamate transporter, is less capable to adhere to and invade A549 respiratory epithelial cells *in vitro*. Furthermore, this mutant showed a decreased virulence in a rat model of infection (29). In *Mycobacterium tuberculosis*, glutamine synthetase GlnA1 is essential for growth in macrophages (31). In another intracellular pathogen, *Salmonella typhimurium*, *glnA* is regulated by the Ntr-system (18). This gene shares an operon with genes encoding the two component system NtrB/C. Mutation in either *glnA*, *ntrB,* or *ntrC* resulted in a marked reduction of virulence and a reduced ability to survive within host cells (18).

In this study, we assessed the importance of glutamine and glutamate metabolism in *S. pneumoniae* by evaluating the contribution of the glutamine / glutamate regulator GlnR and its target genes, *glnP*, *glnA,* and *gdhA*, to pneumococcal virulence. To this end, we investigated the ability of mutants for these genes to adhere to a human pharyngeal cell line *in vitro*. Furthermore, we tested the individual contribution of these genes in three murine infection models, representing the three major phases of pneumococcal disease: colonization, pneumonia, and bacteremia. In addition, we used microarray analysis to examine the global gene expression of the *glnA-glnP* mutant, which was severely affected in virulence.

Materials and Methods

Bacterial strains and media

All strains used in this study were constructed in a *S. pneumoniae* D39 genetic background (NCTC 7466; serotype 2) and are listed in Table 1. The *glnAP* mutant was constructed by transformation of chromosomal DNA from the *glnP* deletion mutant to the *glnA* mutant and selecting for erythromycin resistance (0.25 µg/ml) on GM17 agar plates supplemented with 1% sheep blood (Johnny Rottier, Kloosterzande, The Netherlands). Bacteria were checked for spectinomycin (150 µg/ml) resistance and for the *glnA* mutation by PCR. Stocks were frozen in 10% glycerol and stored at -80°C until further use.

Table 1. Bacterial strains used in this study.

Strain	Mutation	Function	D39 / TIGR4 accession#	Resistance cassette	Reference
Δ*cps*	*cps*	type 2 capsule locus	*spd0315-spd0328*	kanamycin	(26)
Δ*glnR*	*glnR*	glutamine synthetase repressor	*spd0447 / sp0501*	none[a]	(17)
Δ*glnA*	*glnA*	glutamine synthetase	*spd0448 / sp0502*	spectinomycin	(17)
Δ*glnRA*	*glnR, glnA*			[a], spectinomycin	(17)
Δ*glnP*	*glnP*	glutamine/glutamate transporter	*spd1098 / sp1241*	spectinomycin	(17)
Δ*glnAP*	*glnA, glnP*			spectinomycin, erythromycin	this study
Δ*gdhA*	*gdhA*	glutamate dehydrogenase	*spd1158 / sp1306*	erythromycin	(17)

[a] premature stop codon introduced

In vitro adherence

Adherence of pneumococci to epithelial cells was studied essentially as described previously (4, 17). In short, the human pharyngeal cell line Detroit 562 (ATCC CCL-138) was cultured in RPMI 1640 without phenol red (Invitrogen) supplemented with 1 mM sodium pyruvate and 10% fetal calf serum (FCS). Aliquots of bacteria (grown to mid-log in GM17-broth) stored at -80°C were thawed rapidly, harvested by centrifugation, and resuspended in RPMI 1640 medium without phenol red supplemented with 1% FCS to $1x10^7$ colony forming units (CFU) per ml. Monolayers of Detroit 562 in 24-well tissue culture plates were washed twice with 1 ml PBS, and 1 ml of bacterial suspension was allowed to adhere for 2h at 37°C in a 5% CO_2 atmosphere. Non-adherent bacteria were removed by three washes with 1 ml PBS, after which the Detroit 562 cells were detached by adding 200 µl of trypsin/EDTA and lysed by the addition of 800 µl of ice-cold 0.025% Triton X-100 in PBS. Serial 10-fold

dilutions were plated on blood agar plates to count the number of adherent bacteria, and corrected to account for small differences in count in the initial inoculum. All experiments were performed in triplicate and repeated at least three times. Significant differences between wild-type and mutant strains were determined using the Mann Whitney U test ($P<0.05$). Wild-type and mutants strains grew comparably in RPMI medium (without phenol red supplemented with 1% FCS) alone, without extra addition of glutamine.

Experimental virulence in mice

Infection models were essentially performed as described previously (9, 10, 15). Nine-week old female outbred CD-1 mice (Harlan, Horst, Netherlands) were used for all infection models. Prior to the infection experiments, D39 wild-type and mutants were passaged in mice as described previously (15). Cultures of mouse-passaged *S. pneumoniae* strains were grown to an optical density at 600 nm of 0.3, and stored in aliquots at -80˚C in 10% glycerol. Prior to infection, these aliquots were spun down and bacteria were resuspended in sterile PBS to 10^6 CFU in volumes depending on the infection model used. Upon intranasal infection, mice were anesthetized with 2.5% (vol/vol) isoflurane / O_2. At predetermined time points after infection depending on the infection model used, groups of mice were sacrificed by cervical dislocation and samples of various sites were taken to determine the bacterial load. During infection, signs of disease were closely monitored. If animals reached a moribund state, they were sacrificed by cervical dislocation and excluded from the experiment prematurely. All animal experiments were performed with approval from the Animal Experimentation Committee (DEC) of Erasmus Medical Centre, Rotterdam, The Netherlands.

Colonization model of infection

In the colonization model, 10 µl of PBS containing 10^6 CFU of D39 wild-type or mutant was administered to the nostrils of groups of five mice as described previously (14). Due to this small volume, only the nose (nasopharynx) of the mice becomes infected. Bacteria were recovered from the nasopharynx by flushing the nose with 2 ml sterile PBS (16), and lungs were removed from the body and homogenized in 2 ml of sterile PBS using a hand held homogenizer (polytron PT 1200, Kinematica AG). Viable bacteria from the nasal lavage fluid, homogenized lungs, and blood samples were quantified by plating serial 10-fold dilutions on Colombia blood agar (Oxoid) supplemented with 5% (vol/vol) defibrinated sheep blood (Biotrading). Time points for sampling were 30 min, 24h, 48h, 96h, and 192h post-infection. The 30-min time point is considered to be the start of the infection, and is therefore

referred to as t=0. Bacteriology results are expressed as geometric mean ± standard errors of the mean (SEM). Comparison of bacterial loads in the time-course experiment was performed using a Student's *t* test with $P < 0.05$ considered statistically significant.

Pneumonia model of infection

In the pneumonia model, five mice per group were infected with 50 µl of PBS containing 10^6 CFU of pneumococci. Bacteria were recovered from the different sites as described above, with the addition of a blood sample obtained by cardiac puncture. Viable bacteria isolated from the nasal wash, homogenized lungs, and blood were quantified as described above. Time points for sampling were 0, 12, 24, and 36h post-infection. Bacteriology results are expressed as geometric mean ± SEM. Comparison of bacterial loads in the time course experiment was performed using a Student's *t* test with $P < 0.05$ considered statistically significant.

Bacteremia model of infection

In the bacteremia model, groups of six mice were infected in a tail vein with 10^6 CFU resuspended in 100 µl of sterile PBS. Bacteria were recovered from the blood by a lateral tail vein puncture from the same mouse at three predetermined time points after infection (0, 12, 24h) and by a cardiac puncture at the last time point, 36h. In addition, mouse survival times were scored. Two separate experiments were carried out, the first being three groups of mice infected with D39 wild-type, Δ*glnA*, and Δ*glnR*, and the second four groups of mice infected with, again, D39 wild-type, Δ*glnP*, Δ*glnAP*, and Δ*gdhA*. Data of the two experiments were combined, after which analysis of survival times was performed using the log-rank test with $P < 0.05$ considered statistically significant.

DNA microarray analysis

Microarray analysis was performed as described previously (9, 10). In short, cultures of D39 wild-type and Δ*glnAP* were grown in GM17 in static flask cultures at 37°C. Cells were harvested when they reached an optical density at 600 nm of 0.3 (mid-exponential growth phase). Total RNA was isolated from both cultures as described previously, and used to generate fluorescent DNA probes by indirect labeling using standard methods (9, 10). After overnight hybridization, dual-channel array images were acquired with a GeneTac LS IV confocal laser scanner (Genomics Solutions Inc.) and analyzed with ArrayPro 4.5 software (Media Cybernetics Inc.). Spots were screened visually to identify those of low quality. Slide

data were processed using MicroPreP as described (9, 10, 32). Prior to analysis, automatically and manually flagged spots and spots with very low background subtracted signal intensity [5% of the weakest spots (sum of Cy3 and Cy5 net signals)], were filtered out of all datasets. Net signal intensities were calculated using a grid-based background subtraction. A grid-based Lowess transformation was performed for slide normalization, negative and empty values were removed, and outliers were removed by the deviation test. Further analysis was performed using a Cyber-T Student's t test for paired data (22). For identification of differentially expressed genes, only genes with a minimum of six reliable measurements, a Bayesian p-value < 0.001, a False Discovery Rate (FDR) < 0.05, and a standard deviation < ratio were included. Since these criteria are purely a statistical measure of differential gene expression and reproducibility across replicates, an additional fold-change cut-off of 2 was applied.

Accession numbers

The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under GEO Series accession number GSE9850.

Results

Contribution of the GlnR-regulon to *in vitro* adherence

To assess the contribution of glutamine and glutamate metabolism to pneumococcal virulence, directed mutants were created in *glnR* and genes regulated by GlnR. Deletions had no effect on *in vitro* growth, except for the *glnA*-mutant and the *glnA-glnP* double mutant. These strains displayed slower growth in GM17-broth, but upon addition of 0.5 mg/ml glutamine normal growth was restored. Similar observations were made during growth in THY-broth (data not shown).

Pneumococcal colonization is mediated by adherence to respiratory epithelial cells. To assess the relevance of genes of the GlnR-regulon in the process of adhesion, we performed *in vitro* adherence assays using the individual mutant strains. Since unencapsulated strains tend to display significantly higher levels of adherence than encapsulated strains (4), all mutants were constructed in a capsule-negative isogenic derivative of D39 (D39*Δcps*) (26). Adherence capacity of the Δ*glnA*, Δ*glnR*, Δ*glnRA,* and Δ*glnP* strains has been described previously (17), and is included in this study for completeness of analysis. All strains, except Δ*glnR*, showed a severe reduction in adherence of over 50% ($P<0.05$, Fig. 2A and B). For the *glnA*-mutant and the *glnR-glnA* double mutant, adherence was approximately 44% of that of the wild-type. This effect was most likely caused by the *glnA* mutation, as the *glnR* mutant adhered at wild-type levels. The number of adherent *glnP*- and *glnA-glnP* double mutants was approximately 25% of adherent wild-type bacteria. In this case, this appeared to be mainly the result of the lack of *glnP*, as the *glnA* mutant adhered at significantly higher levels, i.e., 44% of wild-type adherence. Finally, deletion of *zwf* and *gdhA* also impaired adherence to Detroit cells (27% and 20% of wild-type, respectively).

Contribution of the GlnR-regulon to colonization

In the colonization model, extended colonization of the nasopharynx without development of invasive disease is achieved by a small-volume inoculum. Indeed, wild-type pneumococci were able to colonize the murine nasopharynx for a period of 192h (Fig. 3). The level of colonization was fairly consistent for 48h, i.e., 10^4 CFU/ml, after which the colonization level slowly started to decrease to 10^3 CFU/ml at 192h.

The colonization kinetics of the *glnR*-mutant did not differ significantly from wild-type during 192 h of colonization (Fig. 3A). In contrast, the *glnA*-mutant displayed a clear

attenuated phenotype. After 48h throughout 96h of infection, mice infected with the *glnA*-mutant had significantly lower bacterial loads in the nasopharynx (Fig. 3A). At 192h the difference was still present, but it was not statistically significant.

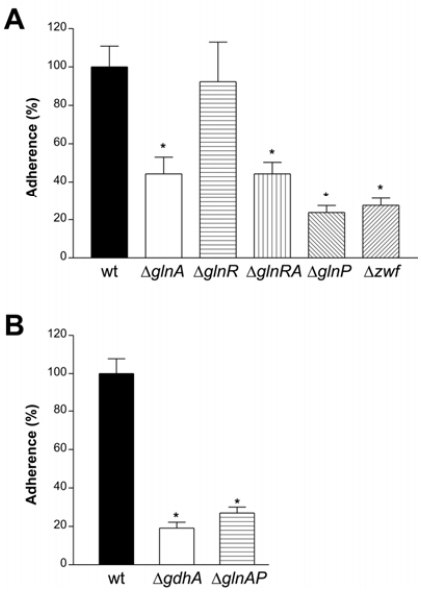

Figure 2. *In vitro* adherence of pneumococcal mutants to the human pharyngeal epithelial cell line Detroit 562. All strains were constructed in a D39*Δcps* genetic background. The adherence of the mutants is given as the percentage relative to the wild-type. * indicates $P < 0.05$.

Neither the *glnP*-mutant nor the *gdhA*-mutant was significantly attenuated during 192h of colonization, whereas the *glnA*-*glnP* double mutant was significantly attenuated after 24h of colonization and severely from 48h onwards to the point of being cleared at 192h. Due to a relatively low colonization level of the wild-type at 96h, no significant difference was reached at this time point (Fig. 3B). The more severe attenuation of the *glnA*-*glnP* double mutant in comparison to that of the *glnA*-mutant is suggestive of an additive effect of the *glnP* mutation to the colonization phenotype of Δ*glnA*.

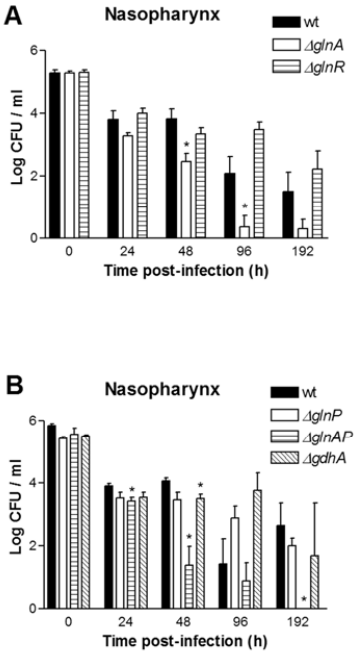

Figure 3. Colonization model. Bacterial loads in the nasal lavage fluid of mice infected with **(A)** D39 wild-type, Δ*glnA* or Δ*glnR* and, **(B)** D39 wild-type, Δ*glnP*, Δ*glnAP*, and Δ*gdhA*. * indicates $P < 0.05$.

Contribution of the GlnR-regulon to pneumonia

In the pneumonia model the infection is monitored at three distinct sites, i.e., the nasopharynx, the lungs, and the blood compartment. This model allows assessment of the two ends of the spectrum of pneumococcal infection, i.e., the progression from nasopharyngeal colonization to invasive disease.

In agreement with the colonization model, we observed that in the pneumonia model, mice infected with the *glnA*-mutant or the *glnA*-*glnP* double mutant had lower numbers of bacteria in the nasal lavage fluid at 36 post-infection than wild-type infected mice, while bacterial loads in mice infected with Δ*glnR,* Δ*glnP* or Δ*gdhA* did not differ from wild-type infected mice (data not shown).

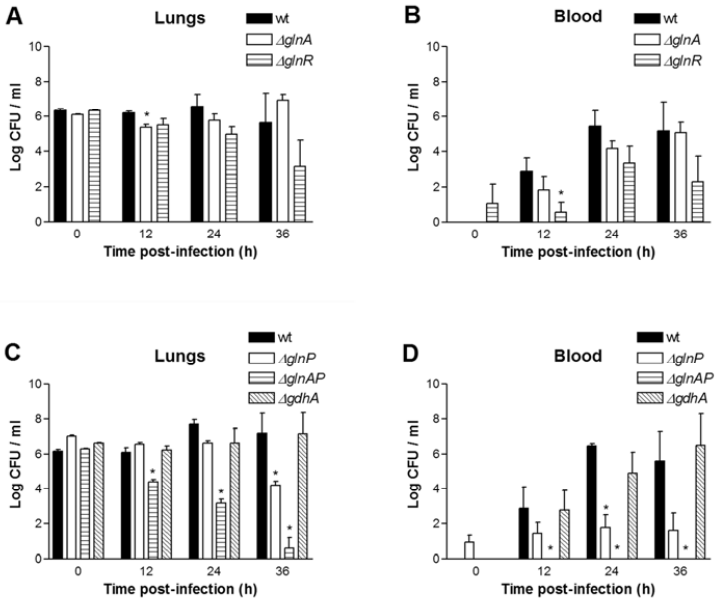

Figure 4. Pneumonia model. Bacterial loads in lungs and blood of mice infected with D39 wild-type, Δ*glnA*, Δ*glnR*, Δ*glnP*, Δ*glnAP*, or Δ*gdhA*: **(A) and (C),** homogenized lungs; **(B) and (D)**, blood. * indicates $P < 0.05$.

During the 36-h infection period, we observed no significant differences between bacterial loads in the lungs of mice infected with the *glnR*-mutant or wild-type bacteria (Fig. 4A). Moreover, the *glnR*-mutant was equally capable to enter the blood component after 12h and onwards (Fig. 4B). Similarly, we did not observe significant differences between the *glnA*-mutant and wild-type, although the bacterial loads in lungs and blood tended to be higher than for the *glnR*-mutant at later time points. However, this difference did not reach statistical significance (Fig. 4A). The *glnP*-mutant was attenuated after 36h of infection in the lungs, indicating that GlnP is required for full virulence in this model (Fig. 4C). In addition, in Δ*glnP*-infected mice fewer bacteria reached the systemic circulation, suggesting that GlnP plays a role in the dissemination of *S. pneumoniae* from the lungs to the blood stream (Fig. 4D). Bacterial counts of the *glnA*-*glnP* double mutant decreased in time, and were significantly lower from 12h post-infection onwards compared to the wild-type (Fig. 4C). Moreover, no bacteria reached the blood stream in mice infected with the *glnA*-*glnP* double mutant (Fig. 4D), again indicating a role for GlnP in transition from the lungs to the blood, with an additive effect of GlnA. The *gdhA*-mutant showed no difference in bacterial loads compared to wild-type in either lungs or systemic circulation (Fig. 4C and D), indicating that this gene is not required for full virulence in this model.

Contribution of the GlnR-regulon to bacteremia

In the bacteremia model, infection of the blood stream is followed for 36h, allowing tracking of bacterial survival and growth within individual mice. Moreover, comparison of results obtained with the blood compartment in the pneumonia model enabled us to discriminate between attenuation in bacterial survival in blood or in the capability to disseminate from the lungs to the blood component. As a measure of disease potential, we compared the mean survival times of mice infected with the different strains.

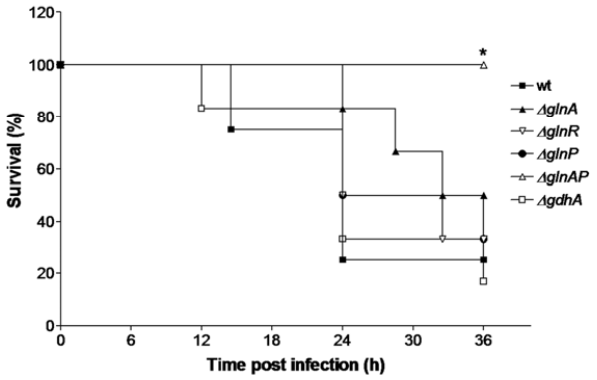

Figure 5. Bacteremia model. Survival of mice infected with D39 wild-type, Δ*glnA*, Δ*glnR*, Δ*glnP*, Δ*glnAP* and Δ*gdhA*. * indicates $P < 0.05$.

Mice infected with D39 wild-type bacteria had a calculated median survival time of 24h, whereas Δ*glnR*- and Δ*glnA*-infected mice had calculated median survival times of 28.25h and 34.25h, respectively (Fig. 5). These differences in median survival times were not statistically different from that of wild-type infected mice. In addition, survival times of mice infected with Δ*glnP* (i.e., 30h) and Δ*gdhA* (i.e., 24h) were not significantly different from wild-type. However, all of the mice infected with the *glnA-glnP* double mutant survived until the end of the experiment (36h), indicating reduced virulence of the double mutant during bacteremia (Fig. 5). Furthermore, all bacteria were cleared from the blood stream after 24h (data not shown).

Expression profile of the *glnA-glnP* double mutant

To investigate the effect of the concurrent deletion of GlnA and GlnP on global gene expression, we performed microarray analysis of D39 wild-type and Δ*glnAP*.

Twelve genes were downregulated in the *glnAP* double mutant. In addition to the GlnR-regulated *glnA*, *glnP,* and *glnQ*, these included genes encoding transcriptional regulators (e.g., spd0096, spd1524), ABC-transporters (e.g., spd1526, spd1607), the starving cell induced protein Dpr, and the serine protease HtrA (Table 2).

Approximately 80 genes were upregulated in the *glnA-glnP* double mutant, most of them involved in amino acid metabolism (Table 2). Strikingly, many of these genes belong to the CodY-regulon, such as the Ami-operon, the Ilv-operon and the Liv-operon (9). Also *gdhA*, regulated by both GlnR and CodY, was strongly upregulated. Interestingly, two putative transcriptional regulators (spd0144 and spd0361) were upregulated, which suggests that other regulatory systems are active to complement the *glnA* and *glnP* mutations.

Of note, besides the genes within the GlnR-regulon, no overlap in differentially expressed genes of the *glnA* mutant (described in reference 17) and the *glnAP* double mutant was observed.

Table 2. Differentially expressed genes in D39 Δ*glnAP*.

Gene identifier				
D39	**TIGR4/R6[a]**	**Gene name**	**Annotation**	**Ratio[b]**
spd0051	sp0044	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	1.1
spd0111	spr0103		argininosuccinate lyase	1.1
spd0142	sp0139		hypothetical protein	1.3
spd0143	spr0139		UDP-glucose 6-dehydrogenase	1.9
spd0144	sp0141	*mutR*	transcriptional regulator	2.0
spd0145	sp0142		hypothetical protein	3.8
spd0146	sp0143		CAAX amino terminal protease family protein	3.9
spd0147	sp0144		CAAX amino terminal protease family protein	4.0
spd0148	sp0145		ABC transporter, substrate-binding protein	3.9
spd0149	sp0146		hypothetical protein	1.7
spd0161	sp0159		hypothetical protein	3.9
spd0283	sp0310		PTS system, IIC component	1.5
spd0300	sp0327		hypothetical protein	1.5
spd0334	sp0366	*aliA*	oligopeptide ABC transporter	2.1
spd0335	sp0368		cell wall surface anchor family protein	1.4
spd0361	sp0395		transcriptional regulator, putative	1.4
spd0364	spr0361		ABC-type polar amino acid transport system	3.5
spd0404	sp0445	*ilvB*	acetolactate synthase large subunit	3.2
spd0405	sp0446	*ilvN*	acetolactate synthase, small subunit	3.4
spd0406	sp0447	*ilvC*	ketol-acid reductoisomerase	3.1
spd0407	sp0448		hypothetical protein	2.5
spd0408	sp0449		hypothetical protein	2.7
spd0409	sp0450	*ilvA*	threonine dehydratase	2.7
spd0424	sp0474		PTS system, cellobiose-specific IIC component	1.1
spd0541	spr0470	*blpO*	bacteriocin	2.4
spd0652	sp0749	*livJ*	branched-chain amino acid ABC transporter	1.0
spd0653	sp0750	*livH*	branched-chain amino acid ABC transporter	1.4
spd0654	sp0751	*livM*	branched-chain amino acid ABC transporter, permease protein	1.7
spd0655	sp0752	*livG*	branched-chain amino acid ABC transporter	1.7
spd0656	sp0753	*livF*	branched-chain amino acid ABC transporter	1.7
spd0749	sp0856	*ilvE*	branched-chain amino acid aminotransferase	1.8
spd0751	sp0858		hypothetical protein	1.8
spd0752	sp0859		hypothetical protein	1.6
spd0753	sp0860	*pcp*	pyrrolidone-carboxylate peptidase	1.7
spd0778	spr0786		hypothetical protein	2.4
spd0778	sp0882		hypothetical protein	2.3
spd0780	sp0884		hypothetical protein	2.0
spd0780	spr0788		hypothetical protein	1.9
spd0781	sp0885		hypothetical protein	2.3
spd0844	sp0955	*celB*	competence protein	1.1
spd0900	sp1013	*asd*	aspartate-semialdehyde dehydrogenase	2.0
spd0901	sp1014	*dapA*	dihydrodipicolinate synthase	2.1
spd1004	sp1119	*gapN*	glyceraldehyde-3-phosphate dehydrogenase, NADP-dependent	2.2
spd1011	sp1126	*glxK*	glycerate kinase	1.1
spd1156	spr1179		putative iron-dependent peroxidase	1.0
spd1158[c]	**sp1306**	***gdhA***	**NADP-specific glumatate dehydrogenase**	2.1
spd1190	sp1356		Atz/Trz family protein	1.1
spd1191	sp1357		ABC transporter, ATP-binding/permease protein	1.1
spd1192	sp1358		ABC transporter, ATP-binding/permease protein	1.3
spd1276	spr1302		hypothetical protein	1.3
spd1464	sp1651	*psaD*	thiol peroxidase	1.3
spd1472	sp1659	*ileS*	isoleucyl-tRNA synthetase	1.1
spd1500	sp1688		ABC transporter, permease protein	1.1
spd1563	spr1598		dicarboxylate/amino acid:cation (Na+ or H+) symporter	1.3
spd1564	sp1754		hypothetical protein	1.1
spd1585	spr1620		ABC transporter, sugar-binding protein	1.7
spd1596	sp1811	*trpA*	tryptophan synthase, alpha subunit	1.1

spd1602	sp1817	*trpE*	anthranilate synthase component I	1.1
spd1649	sp1869	*fatD*	iron-compound ABC transporter, permease protein	1.5
spd1650	sp1870	*fatC*	ABC transporter membrane-spanning permease - ferric iron transport	1.5
spd1651	sp1871	*fecA*	ABC transporter ATP-binding protein - ferric iron transport	1.1
spd1652	sp1872	*fatB*	iron-compound ABC transporter, iron-compound-binding protein	1.1
spd1667	sp1887	*amiF*	ABC transporter ATP-binding protein - oligopeptide transport	1.1
spd1668	sp1888	*amiE*	ABC transporter ATP-binding protein - oligopeptide transport	1.3
spd1669	sp1889	*amiD*	ABC transporter membrane-spanning permease - oligopeptide transport	1.2
spd1670	sp1890	*amiC*	ABC transporter membrane-spanning permease - oligopeptide transport	1.1
spd1783	sp1986		hypothetical protein	1.1
spd1840	sp2031		predicted Zn-dependent hydrolases of the beta-lactamase fold	1.3
spd1845	sp2036		PTS system, ascorbate-specific IIA component, PTS-EII	1.3
spd1954	sp2125		hypothetical protein	2.2
spd1981	spr1961		hypothetical protein	1.1
spd1985	sp2157	*adh2*	alcohol dehydrogenase, iron-containing	1.0
spd1988	sp2160		hypothetical protein	1.1
spd1994	sp2166	*fucA*	L-fuculose-phosphate aldolase	1.2
spd0096	sp0100		transcriptional regulator, PadR family protein	-1.0
spd0381	sp0418	*acp*	acyl carrier protein	-1.0
spd0448[c]	**sp0502**	***glnA***	**glnA, glutamine synthetase**	-6.5
spd0803	sp0910		hypothetical protein	-1.1
spd1098[c]	**sp1241**	***glnP***	**glutamine/glutamate transporter**	-6.2
spd1099[c]	**sp1242**	***glnQ***	**glutamine/glutamate transporter**	-3.2
spd1402	sp1572	*dpr*	starved cells-like peroxide resistance protein	-1.1
spd1524	sp1714		transcriptional regulator, GntR family protein	-1.3
spd1526	spr1560		hypothetical protein (ABC-2 transporter)	-1.1
spd1526	sp1715		hypothetical protein, ABC-2 type transporter	-1.2
spd1607	sp1824		ABC transporter, permease protein (Fe(III))	-1.2
spd2068	sp2239	*htrA*	serine protease	-1.1

[a] Gene identifier as deposited in the NCBI Gene Expression Omnibus (GEO) database under GEO Series accession number GSE9850 (TIGR4, sp; R6, spr).

[b] Microarray ratio is given as log2-transformed expression of Δ*glnPA* / expression of wild-type.

[c] Genes directly regulated by GlnR are indicated in bold fonts.

Discussion

The ability to adequately adapt to changes in the availability of nutrients is a prerequisite for bacterial survival. Several studies have suggested that nitrogen metabolism, and especially glutamine metabolism, is important for virulence of pathogens (18, 29, 31). The transcriptional repressor GlnR regulates, together with glutamine synthetase GlnA, genes involved in glutamine / glutamate uptake and conversion in *S. pneumoniae*, *L. lactis,* and *B. subtilis* (7, 17, 19). DNA-binding assays have demonstrated that GlnR-repression is dependent on GlnA, but the exact mechanism remains unknown (17). As a result, GlnR-targets are derepressed in a *glnA*-mutant. In this study, we investigated the contribution of the transcriptional regulator GlnR and its target genes to pneumococcal virulence, in particular its contribution during colonization, pneumonia, and bacteremia in mice.

Absence of GlnR had no significant effect on bacterial virulence in any of our three infection models used. It could well be that levels of available glutamine at the nasopharynx and in the lungs are considerably low and, hence, no repression by GlnR is triggered. Alternatively, all genes of the GlnR-regulon might be expressed in the Δ*glnR* strain, and redundant expression of these genes might not influence the ability to colonize or cause disease.

The conversion of glutamate to glutamine appears to be required during colonization, as the mutant lacking GlnA was found to be attenuated in colonization of the murine nasopharynx. Alternatively, the lack of GlnA might affect regulation by GlnR through derepression of its gene targets, although this appears less likely given the lack-of-colonization phenotype of the *glnR*-mutant. Attenuation of the *glnA*-mutant was not observed in lungs and in the blood stream during the pneumonia and bacteremia models of infection, indicating that GlnA is not required for bacterial survival in lungs and the transition from the lungs to the blood in our infection models.

GlnP, which is part of the main glutamine / glutamate ABC transporter GlnPQ, is not required for colonization of the murine nasopharynx. Interestingly, GlnP was found to be involved in adherence to Detroit 562 cells, suggesting a role in colonization in humans. It cannot be excluded that the difference between *in vitro* adherence and the murine colonization model reflects the differences between the *in vitro* and *in vivo* setting. For instance, expression of *glnP* might be required in RPMI medium, but not in the murine (or human) nasopharynx. In group B streptococci it has been proposed that *glnQ*, also part of the glutamine / glutamate transporter, is involved in adherence to fibronectin and virulence in rats, possibly by modulating cytoplasmic glutamine levels (29). GlnP was also identified as a

candidate for fibronectin binding using phage display library analysis (1). However, binding of fibronectin by GlnP of streptococci has never been confirmed, suggesting an indirect involvement (1, 29). In the pneumonia model, mice infected with the *glnP*-mutant showed a lower bacterial load in the lungs at 36h than wild-type infected mice. Strikingly, while the number of wild-type bacteria increased with time, fewer *glnP*-mutants reached the blood stream from 24h post-infection onwards. This suggests a role for GlnP in the transition from the lungs to the blood stream. Uptake of glutamine could be of importance at this stage of infection, i.e., entering the blood stream. The impaired glutamine uptake in the *glnP*-mutant might therefore create a growth disadvantage leading to a smaller number of bacteria in the blood circulation. However, we cannot rule out the possibility that the Δ*glnP* phenotype, i.e., impaired transition from lungs to blood, is caused by multiple factors. Fewer bacteria cause less tissue damage in the lungs and this will consequently lead to less spill-over into the blood. One such example might be lower pneumolysin concentrations due to lower bacterial loads in the lungs (11).

The *glnA*-*glnP* double mutant displayed an attenuated phenotype similar to the *glnA*-mutant in the colonization model, most likely caused by the *glnA* mutation only, given the lack-of-colonization phenotype of the *glnP*-mutant. Moreover, since GlnP is part of the main glutamine and glutamate transporter (17), our data suggest that pneumococcus, while colonizing the murine nasopharynx, is able to acquire glutamine and glutamate through other processes such as peptide uptake and degradation. Different results were obtained in the lungs; here the *glnA*-mutation alone did not result in attenuation whereas mutation of both *glnA* and *glnP* did, suggesting that pneumococcus cannot easily use other sources for acquisition of glutamine at this particular site. The double mutant is not able to convert glutamate to glutamine (GlnA), and is not able to take up free glutamate or glutamine (GlnP). Consequently, the double mutant has to rely on another system for acquiring these amino acids. If so, such a system is apparently not able to complement the lack of GlnA and GlnP sufficiently when pneumococci reach the lungs or blood circulation, in contrast to the nasopharynx, where only glutamine synthesis (GlnA) contributes to survival. The *glnAP* mutant was able to grow in defibrinated blood *in vitro*, but this growth was slower and to a lower cell density than the wild-type. Addition of glutamine rescued the phenotype of Δ*glnAP* to wild-type growth (data not shown). This suggests that, when added in high concentrations, glutamine can enter the cell, possibly by other uptake systems with very low affinity for glutamine or by passive diffusion.

The microarray data showed that predominantly genes involved in amino acid metabolism are upregulated in the *glnAP* mutant. These genes, most of them belonging to the CodY regulon (9), are probably upregulated because the double mutant is starved for amino acids, glutamine in particular. Although glutamine was present in the medium used for the expression study, the gene regulatory network sensing and controlling general amino acid metabolism seemed perturbed. This is underscored by the differential expression of four transcriptional regulators, however, their function is yet unknown. This suggests that regulation of amino acid metabolism within the pneumococcal cell is quite complex, and has severe effects on fitness and virulence. The impact on virulence could also be explained by the twofold downregulation of the expression of *htrA* in the *glnAP* mutant, as it was shown previously that this gene is required for virulence in D39 (12).

The gene *gdhA*, encoding glutamate dehydrogenase, does not appear to be required for virulence at any site in our infection models. No paralog of *gdhA* is present in the pneumococcal genome, which could complement its metabolic function. Regulation of *gdhA* by CodY has been described and might be the principle regulator *in vivo*, by repressing *gdhA* expression (9, 17). Possibly, *gdhA* expression is coordinated in such way that it is only activated in specific conditions (e.g., nitrogen limitation), and that these conditions are not encountered by the pneumococcus in our mouse models.

We have described the behavior of mutants lacking genes of the GlnR-regulon, the regulatory system of glutamine and glutamate metabolism, during adherence to human pharyngeal epithelial cells *in vitro* and experimental virulence in mice. We have identified two genes that play a role in virulence, namely *glnA* and *glnP*. The gene *glnA* encodes glutamine synthetase, which is required for colonization, and *glnP* encodes a membrane glutamine / glutamate permease, which is required for survival in the lungs and, possibly, for the transition from the lungs to the blood circulation. This study provides novel insight into the nutritional requirements of *S. pneumoniae* within its host, more specifically the glutamine and glutamate requirements. The different phenotypes of mutants during *in vitro* adherence to human pharyngeal cells and colonization of the murine nasopharynx suggest that different host species (i.e., mice and humans) may have different adhesion properties and nutritional supply for the bacterium.

Many studies have shown that expression of pneumococcal proteins during pathogenesis is required at different stages of infection (15, 21, 25). One study, performed by Oggioni and co-workers, presents evidence that the “transcriptional state” of the pneumococcal cells differs at particular niches within the host (24). The bacterium has to deal

with the supply of nutrients that each site of the body offers. For this reason, co-regulation of site-specific metabolic and virulence factors by nutritional regulators is likely to occur. In this study, we report on the role of the glutamine / glutamate metabolism and its regulation during our experimental virulence models in mice. The site-specific requirements for GlnA and especially GlnP reported in this study might offer novel strategies to explore these molecules as future drug targets that specifically target invasive disease, while leaving colonization unaffected.

In conclusion, genes within the GlnR-regulon are of definite importance for bacterial survival within our experimental models of infection, with different subsets of the genes involved in glutamine / glutamate metabolism contributing to pneumococcal survival during different stages of infection.

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CHAPTER 7

Pneumococcal gene regulatory systems controlling nitrogen metabolism and virulence

Gene regulation and metabolism in *Streptococcus pneumoniae*

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(Manuscript in preparation)

Abstract

Efficient nutritional gene regulation is essential for bacteria in order to sustain in and adapt to their continuously changing niches. Transcriptional regulators play a crucial role in orchestrating gene expression as a reaction to their direct environment. In Gram-positive bacteria, nitrogen metabolism has been studied extensively and key transcriptional regulators have been identified. In addition to metabolic genes, these regulators also affect expression of virulence genes, suggesting that these processes are intertwined in bacteria and tightly controlled to increase fitness while residing in a particular niche. In this review, we discuss the role of two transcriptional regulators, GlnR and CodY, in nitrogen metabolism of Gram-positive bacteria in general, and in the context of virulence in *Streptococcus pneumoniae* in particular.

Introduction

Regulation of gene expression in bacteria is crucial for survival, as specific gene products are required during specific conditions, and therefore need to be expressed and regulated accordingly. Especially in bacterial model organisms like *Escherichia coli* and *Bacillus subtilis*, regulators of gene expression have been investigated extensively. Similarly, various studies have shown that gene regulatory networks control various cellular processes of the human Gram-positive pathogen *Streptococcus pneumoniae*, which are essential for different aspects of pneumococcal pathogenesis. Over the last 10 years, attempts to identify the actual genes involved in these processes have been made in several large-scale studies using a signature-tagged mutagenesis approach (4, 16, 27, 36). These studies have demonstrated the impact of a variety of gene products on the different phases of pneumococcal infection such as colonization and invasive disease, many of which (are predicted to) encode transcriptional regulators. Interestingly, several of these regulatory genes were found to contribute to virulence in a tissue-specific manner. Apart from the known virulence genes, genes involved in basal metabolism appear to have a vital role in pneumococcal pathogenesis as well.

Studies on bacterial metabolism and production of secondary metabolites have elucidated many basal and conserved metabolic pathways, such as glycolysis, gluconeogenesis, and nitrogen assimilation. Especially for bacteria with industrial relevance, this knowledge has been used to improve their industrial application, for instance flavor improvement by the dairy bacterium *Lactococcus lactis*, or increased yields of solvents produced by *Pseudomonas putida* and *Clostridium acetobutylicum*.

For pathogenic bacteria such as *S. pneumoniae*, knowledge of metabolic pathways is still limited. The ability of these bacteria to adapt to specific niches is likely to be reflected in their metabolic pathways and the regulation thereof. The link between these regulatory pathways and bacterial virulence is nowadays increasingly recognized.

In this review we discuss regulation of nitrogen acquisition and metabolism in *S. pneumoniae*, which is predominantly mediated by GlnR and CodY. These regulatory proteins have been studied extensively in *Bacillus subtilis* and *Lactococcus lactis*, and in several other Firmicutes. This group also contains pathogenic species for which GlnR and CodY have been linked directly or indirectly to virulence (31, 35, 45). These studies are discussed in detail, and, when applicable, put in the context of pneumococcal GlnR and CodY. In addition, the role of these regulators and their identified targets in pneumococcal virulence are discussed.

Nitrogen metabolism

Nitrogen is an essential building block for virtually every biological molecule. Bacteria utilize nitrogen from their environment and from a variety of molecules, such as free amino acids, peptides, purines, inorganic nitrogen, and gaseous nitrogen. To be able to acquire sufficient nitrogen and nitrogen-containing molecules, bacteria are equipped with sophisticated systems.

S. pneumoniae also has the ability to use amino acids as nitrogen source (51). According to early studies, growth media need to contain valine, leucine, isoleucine, arginine, asparagine, histidine, and glutamine (1, 43). Interestingly, auxotrophy for amino acids can vary between strains (1, 5, 25, 43).

The vast majority of nitrogen-containing compounds within the cell are derived from the two amino acids glutamine and glutamate. The system dedicated to glutamine and glutamate processing is the so-called GOGAT system. GOGAT has been described in Gram-positive bacteria (6, 10, 19, 26), and it regulates the intracellular glutamine/glutamate concentrations via several enzymes such as glutamine synthase (GS or GlnA) and glutamate synthase (encoded by *gltAB* and *gltBD*). In addition, glutamine and glutamate can be taken up via an ABC-transporter encoded by *glnP* and *glnQ*.

In *B. subtilis* and several other Firmicutes, nitrogen metabolism is regulated by the nitrogen-specific regulators GlnR, TnrA (12, 39, 47, 52, 53), and by the pleiotropic regulator CodY (12, 52).

Nitrogen regulation: GlnR

Recently, the transcriptional regulator GlnR was shown to regulate several genes involved in glutamine and glutamate uptake and inter-conversion in *S. pneumoniae* (24 and **chapter 5**). The gene *glnR* is located upstream of the gene *glnA*, and these genes are transcribed as one transcript. In addition to its own operon, pneumococcal GlnR regulates expression of *gdhA*, and an operon consisting of *glnP*, *glnQ*, and *zwf*. The enzyme GlnA converts glutamate and ammonium in glutamine, while GlnP and GlnQ form a glutamate/glutamine transporter and Zwf functions as a glucose-6-phosphate dehydrogenase (24). The repressing activity of GlnR is dependent on glutamine and ammonium concentrations. Besides this enzymatic function, an important role for GlnA in regulation has also been suggested, since the interaction of GlnA with GlnR is required for the repression of GlnR-targets (24) (Figure 1).

In *B. subtilis*, glutamine synthetase converts glutamate and ammonium to glutamine. Its enzymatic activity is directly inhibited by glutamine. In contrast to GlnR, the gene encoding TnrA is transcribed mono-cistronically. This MerR regulator protein family member binds to specific DNA recognition sites, which, interestingly, overlap with GlnR recognition sites. Sonenshein has recently described a model for regulation by GlnR and TnrA in which the transcriptional regulators TnrA and GlnR, the metabolic enzyme GlnA, and the amino acid glutamine play key roles (47). In this model, feedback-inhibition of GlnA as a result of inhibition by glutamine determines the binding capacity of GlnR and TnrA through direct protein-protein interaction. During nitrogen-limitation, GlnA is enzymatically active, while TnrA binds to its DNA-binding recognition site. Subsequently, TnrA activates or represses genes, while GlnR is not able to bind to the DNA because it cannot dimerize. Conversely, when glutamine is abundantly present, GlnA will be feedback-inhibited as a result of glutamine binding, resulting in inactivation of its enzymatic activity. GlnA will be able to interact with TnrA, which when complexed with GlnA is no longer able to bind to its DNA recognition sequences. In contrast, GlnR in complex with feedback-inhibited GlnA will bind to DNA, thereby repressing gene expression of its targets (47). Recently, supporting evidence for this model was provided by Wray and Fisher (54). Their data suggest that the C-terminal domain of GlnR acts as an auto-inhibitory domain, since truncated versions of GlnR lacking this domain formed significantly more dimers than the full-length proteins.

The genomes of *L. lactis* and *S. pneumoniae* do not contain a homologue of TnrA. Furthermore, we found that the only target that pneumococcal GlnR shares with *B. subtilis* GlnR is the *glnRA* operon (24 and **chapter 5**). In *B. subtilis*, GlnR also regulates the expression of *tnrA* and that of the *ureABC* operon, genes which are not present in *S. pneumoniae* (12, 52). Interestingly, the *ureABC* operon is under strict regulation of GlnR, TnrA and CodY in *B.subtilis*, with CodY being the major repressor (52).

The above suggests that nitrogen regulation in lactic acid bacteria varies considerably from other studied Gram-positive bacteria. For instance, the expression of *gdhA* is regulated by both GlnR and CodY in *S. pneumoniae* (17, 24), while this gene is regulated by CcpA, RocR, and AhrC in *B. subtilis* (3). These differences in nitrogen regulation could be a reflection of the different preferences for particular niches, although the lactic acid bacterial species can inhabit a wide variety of niches.

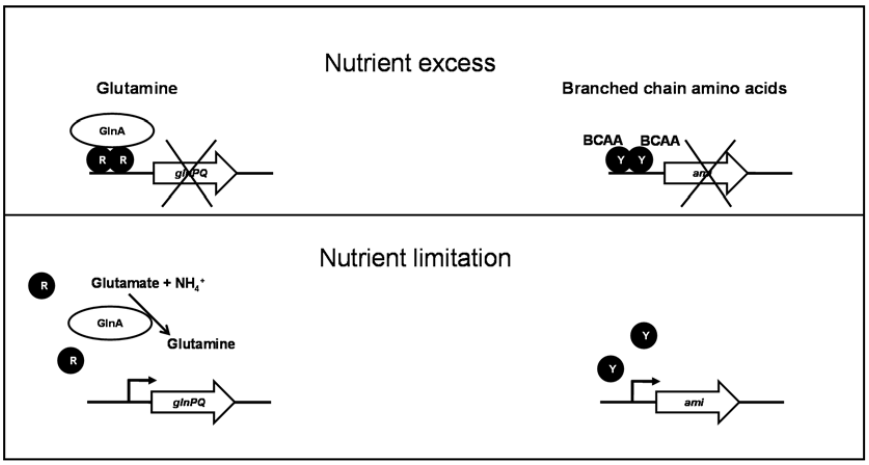

Figure 1. Models of transcriptional regulation by GlnR (R) and CodY (Y). During glutamine, glutamate or ammonium excess, GlnR is in complex with GlnA. This complex binds to the GlnR operator and represses its target genes, *e.g. glnPQ*. When glutamine, glutamate or ammonium levels decrease, GlnR and GlnA dissociate and transcriptional repression is relieved. GlnA is consequently able to catalyze the conversion of glutamate and ammonium in glutamine. During BCAA excess, CodY is associated with BCAAs, and consequently, binds the promoter of its target genes, e.g. the *ami* operon, to repress gene expression. When BCAA levels decrease, CodY-repression is relieved and the target genes will be expressed.

Pneumococcal GlnR and virulence

Recently, we investigated the contribution of the individual genes of the entire pneumococcal GlnR-regulon to *in vitro* adherence and virulence in mice (18 and **chapter 6**). In this study, all genes were found to invariably contribute to adherence of *S. pneumonia* to human pharyngeal epithelial Detroit 562 cells, while in the mouse infection models GlnA was demonstrated to be required for colonization and GlnP for bacterial survival in the lungs (18). Moreover, a *glnA-glnP* double mutant was fully avirulent. Apparently, glutamine/glutamate metabolic genes play a crucial and site-specific role in pneumococcal virulence (18).

Nitrogen regulation: CodY

CodY is a pleiotropic transcriptional regulator of many genes. It represses genes that are typically expressed in conditions of nutrient limitation. Recently, we have shown that pneumococcal CodY regulates a variety of genes involved in several cellular processes, such as iron uptake, carbon metabolism, and, potentially, adherence (17 and **chapter 4**). The most pronounced cellular processes regulated by CodY, however, are nitrogen and amino acid uptake and metabolism (17). All these processes are connected by CodY-regulation, which

makes it possible for the pneumococcus to adequately react to external signals. The specific signals pneumococcal CodY recognizes are the branched-chain amino acids (BCAA) isoleucine, leucine, and valine, leading to an orchestrated change in gene expression in anticipation of a changing environment (Figure 1).

CodY was first described in *B. subtilis* (44), and has been studied extensively in this organism. *B. subtilis* CodY regulates the expression of over a hundred genes, varying from genes involved in sporulation, genetic competence, motility, and chemotaxis, but its role is most pronounced in nitrogen metabolism (32). In *B. subtilis*, CodY reacts on intracellular GTP concentrations and on branched-chain amino acids, and these molecules enhance binding of CodY to its DNA-binding box (38, 42). *B. subtilis* CodY carries a helix-turn-helix and a GTP-binding motif at the carboxy-terminal half of the protein. Strikingly, in *L. lactis*, *Streptococcus mutants* and *S. pneumoniae*, the GTP-binding motif is present, but CodY did not seem to respond to GTP, as *in vitro* binding assays did not show any enhancement of protein-DNA interactions (8, 17, 28). The GTP-binding motifs of *L. lactis*, *S. mutants* and *S. pneumoniae* do show multiple amino acid residue substitutions, which may explain the observed unresponsiveness to GTP (38, 44). The difference of CodY-mediated repression in response to GTP between this group of bacteria and *B. subtilis* might reflect the importance of GTP-sensing in *B. subtilis*: GTP plays a crucial role in the physiology of *B. subtilis*, as pivotal species-specific processes such as sporulation take place when the cellular levels of GTP decrease (29, 33). In contrast, *L. lactis*, *S. mutants,* and *S. pneumoniae* lack a functional sporulation machinery.

Stringent response and CodY

In *B. subtilis*, CodY is clearly linked to the so-called stringent response. The stringent response is a reaction during amino acid limitation in which ribosomal RNA synthesis is shut down and a ribosome-bound protein called RelA is activated. RelA is a GTP pyrophosphokinase that converts GTP to (p)ppGpp, a molecule which is often referred to as the alarmone (for review see 22, 37). During the stringent response the GTP concentration is lowered by two processes: production of (p)ppGpp out of GTP, and the inhibition of IMP dehydrogenase (20, 33). Recently, Lemos *et al.* have shown that in *S. mutants*, CodY-regulation and the stringent response (and the effect of RelA activation) are closely linked (28). The introduction of an additional *codY* mutation in *S. mutans* Δ*relAPQ* fully restored growth in medium lacking leucine or valine, demonstrating that the growth-defective phenotype of Δ*relAPQ* was directly linked to CodY. Moreover, artificially lowering GTP

levels did not change expression of CodY targets in *S. mutans*, indicating once more that *S. mutans* CodY, like CodY of *S. pneumoniae* and *L. lactis*, is not responsive to GTP. However, the exact molecular link between the (p)ppGpp-mediated stringent response and CodY in lactic acid bacteria remains unclear.

CodY and intracellular signaling of peptides

The first described target of CodY is the dipeptide permease of *B. subtilis* encoded by *dppABCDE* (40). Similarly, the *L. lactis opp* gene, coding for the oligopeptide permease Opp, is regulated by CodY (8, 9, 15). Uptake of oligopeptides in *S. pneumoniae* is mediated through an ABC transporter, the Ami/Ali permease, which is encoded by *amiACDEF*, *aliA*, and *aliB* (2). Interestingly, the Ami/Ali permease has been shown to be involved in adherence, by either direct interaction to human receptors or modulating pneumococcal adhesins (7). Moreover, the Ami/Ali permease is required for successful colonization of the nasopharynx of mice, but not for invasive infection (23).

Claverys and co-workers hypothesized that peptide uptake by the Ami/Ali permease results in an intracellular response (5). In their model, uptake of peptides modifies the intracellular amino acid pool, which will activate a global regulatory protein, which in turn will activate (or repress) gene expression. Interestingly, this route also included a direct branch towards the stringent response, i.e., the response to low peptide or amino acid pools. Recently, we showed that the genes encoding the Ami/Ali permease are part of the pneumococcal CodY-regulon (17). This suggests that the expression of the *ami* operon and *ali* genes is regulated in response to amino acid availability, BCAAs in particular, conferring a negative feed-back. Direct molecular links with the stringent response have as yet not been identified, and therefore, direct molecular signaling between these systems seems unlikely.

CodY and carbon metabolism

In *B. subtilis*, CodY also plays a profound role in carbon metabolism. For instance, the gene encoding acetate kinase (*ackA*) is activated by CodY. This activation ensures that the cell has at least one pathway operational to generate ATP during nutrient excess (41). In a recent review by Sonenshein, an extensive regulatory scheme has been described in which CcpA and CodY act as master regulators of carbon flow in *B. subtilis* (46). In addition, GlnR and TnrA also play a role in balancing the flow of metabolites, but their roles are more specific for nitrogen metabolism.

A CcpA homologue exists in the pneumococcus, designated RegM originally (13). Giammarinaro and Paton found that this regulator represses the expression of two galactosidases and that *regM* mutants have reduced transcription of the capsular polysaccharide locus. In addition, they observed that RegM does not mediate the repression of these genes by glucose, as is found in other bacteria. Moreover, *regM* mutants were severely attenuated compared to wild-type in murine virulence studies (13). In line with this, a more recent study described that a pneumococcal mutant in the *ccpA* homologue has no effect on catabolite repression by glucose, and that this mutant is also severely attenuated in virulence (21).

Secondary metabolite formation by *S. pneumoniae* in the presence and absence of oxygen has been described by Taniai and coworkers (50). During anaerobic growth, it is thought that pneumococcus mainly produces lactate (homolactic fermentation), as glucose is used to produce lactate through pyruvate. However, during aerobic growth acetate is also produced from pyruvate in order to expand the ATP pool. The pneumococcus produces L-lactate oxidase encoded by *lox*, which requires lactate to produce pyruvate and H_2O_2, which in turn is a substrate for production of acetate and for raising ATP levels. Remarkably, this enzyme is not often found in other lactic acid bacteria, which could explain the specificity of pneumococcus for its particular niche. Pyruvate oxidase encoded by *spxB* produces acetyl phosphate and H_2O_2 from pyruvate. Spellerberg and coworkers (49) have tested a *spxB*-deficient mutant in *in vitro* growth, adherence assays, and murine infection models, and observed that this mutant was unable to grow aerobically in chemically defined medium unless supplemented with acetate. This finding underlines the importance of acetate to create acetyl phosphate, a precursor of amino acids and fatty acids, during *in vitro* growth. Moreover, the *spxB* mutant adhered at lower levels to the human pharyngeal cell line Detroit 562 unless supplemented with acetate, suggesting that production of acetyl phosphate or possibly other secondary metabolites is essential for regulation of adherence factors. Finally, SpxB was shown to be required for full virulence, in particular prolonged nasopharyngeal colonization, bacterial replication in the lungs, and translocation to the bloodstream (34, 49). The exact role for pneumococcal CodY in regulation of carbon metabolism and the production of secondary metabolites remains to be established.

Pneumococcal CodY and virulence

The main role of CodY in pneumococcal virulence is at the early stages of infection, i.e., adherence and colonization, as we showed in **chapter 4** (18). Mutants lacking *codY* are

severely affected in their ability to adhere to human epithelial Detroit 562 cells. These mutants are also severely reduced in the ability to colonize the murine nasopharynx (17).

In a recent paper, the transcriptional response of *S. pneumoniae* upon contact with human epithelial cells was described (48). In this study, microarray analysis revealed that the transcriptional activity of many genes is changed when pneumococcal cells interact with epithelial cells. Interestingly, some pronounced CodY targets were among these genes. For example, the genes *ilvB*, *ilvE*, and *asd* (encoding acetolactate synthase, branched chain amino acid aminotransferase, and aspartate semi-aldehyde dehydrogenase, respectively), were strongly downregulated. This is in agreement with our observations that the *codY* mutant is severely affected in its ability to adhere to the Detroit 562 epithelial cells (17). In the *codY* mutant, *ilvB*, *ilvE*, and *asd*, were strongly upregulated during *in vitro* growth. This upregulation might have had a negative effect on the adherence ability.

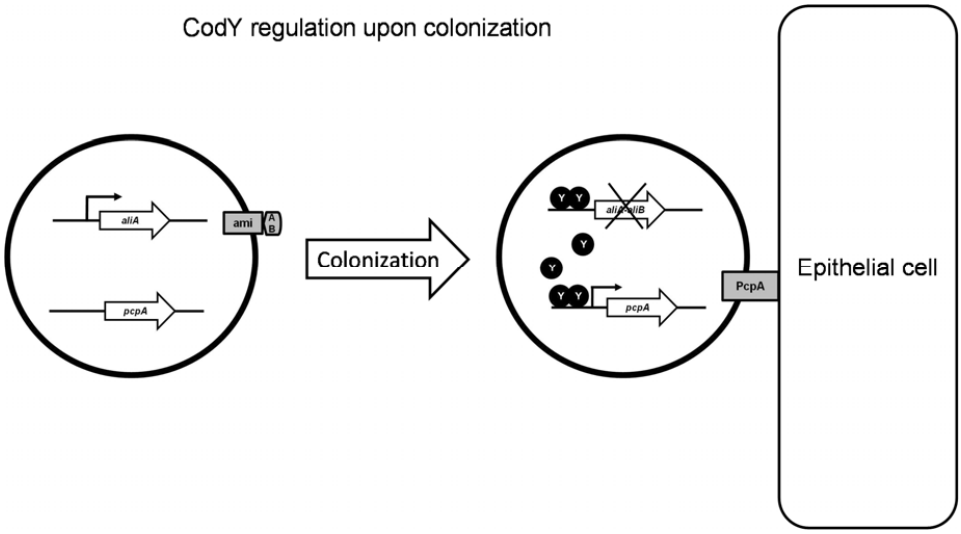

Figure 2. Model of transcriptional regulation by CodY (Y) during colonization. During contact with epithelial cells CodY-regulation is required for the expression of PcpA, a putative adhesin. Expression of other genes in the CodY-regulon, e.g. *aliA* and *aliB*, will be repressed.

The gene *pcpA*, encoding a choline binding protein, is one of the genes that is directly regulated by CodY (17). Of note, although CodY usually represses its targets, it appears to directly activate expression of *pcpA*, as PcpA was downregulated in the *codY* mutant and CodY interacts with the promoter. Interestingly, the *pcpA* mutant was similarly affected in adherence as the *codY* mutant, and no additive phenotype was observed in the *pcpA-codY*

double mutant, suggesting direct regulation of *pcpA* expression by CodY during colonization (Figure 2). Hence, the absence of an additive phenotype is suggestive for a direct mechanism for adherence through CodY, activating expression of *pcpA*, which in turn facilitates adherence to epithelial cells (17). In agreement with this, recent studies show that *psaA* and *pcpA* were more highly expressed *in vivo* (i.e., in the murine nose, lungs, and blood) than *in vitro* (Todd-Hewitt Yeast broth) (30, 34). However, Glover *et al.* described that PcpA is not expressed during colonization, due to PsaR repression (triggered by high Mn^{2+} concentrations in the nasopharynx), and is mainly involved in invasive disease (14). In addition, preliminary data by Bootsma *et al.* shows that expression of *pcpA* in TIGR4 is lower in nasopharyngeal lavage fluid than in *in vitro* culture medium, and expression in blood is comparable with expression in *in vitro* cultures (Bootsma *et al.*, personal communication). Additional data confirming this expression data, is that a *pcpA* knockout mutant colonizes at wild-type levels (Cron *et al.*, personal communication) (Fig. 3). This discrepancy might be caused by the use of different pneumococcal strains and different murine models of infection. In conclusion, the exact role of PcpA during pneumococcal disease remains unclear. It is clear that CodY plays a profound role in the cellular physiology of *S. pneumoniae*, but the exact molecular mechanisms of its involvement in adherence and colonization remain unclear.

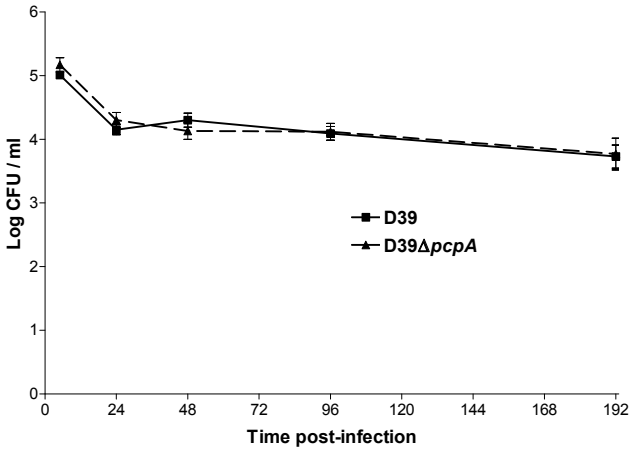

Figure 3. Bacterial loads in the nasal lavage fluid of mice intranasally challenged with with 10^6 CFU of either D39 wild-type or D39Δ*pcpA* (Bootsma et al., unpublished).

CodY has been shown to play a pronounced role in regulating virulence in other Gram-positive bacteria, among which *S. pyogenes*, *S. mutants*, *Listeria monocytogenes*,

Enterococcus faecalis, Bacillus anthrasis, Clostridium difficele, and *Staphylococcus aureus* (11, 31, 35, 45 and references therein). In these bacteria, CodY is required for repression of genes directly involved in virulence (*e.g.*, regulators activating toxin production) or processes closely related to virulence (*e.g.*, biofilm formation).

Concluding remarks

Strikingly, so far CodY and GlnR have only been described as important regulators in bacteria of the Firmicutes group. Interestingly, these bacteria live in various niches such as soil, dairy, skin, oral cavity, blood, and the respiratory tract. These niches differ in many aspects, for instance temperature, nutrient supply, osmotic pressure, and pH, but also in the host immune response in case of commensals and pathogens. It will be of interest to investigate whether the regulatory networks controlling basal metabolism of these bacteria provide the basis for the differences in success to sustain in and adapt to their different niches.

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CHAPTER 8

Summarizing discussion

Being one of the major asymptomatic inhabitants of the human upper respiratory tract, *Streptococcus pneumoniae* is also one of the major pathogens to cause various infections, ranging from otitis media (middle ear infection) to bacteremia (blood infection). The currently available 7-valent conjugate vaccine directed against the polysaccharide capsule is effective but has the major drawback of only protecting against a select group of serotypes (defined by their different composition of polysaccharide capsule). Consequently, a considerable part of recent pneumococcal research focuses on identifying suitable protein targets as vaccine antigens. However, this approach is dependent on expression of such proteins at the site of infection. For this reason, the following questions need to be addressed: Which proteins are expressed? When are these proteins expressed? How are the genes encoding these proteins regulated under different conditions? Which nutrients are used when the bacterium resides, for instance, in the nasopharynx or in the lungs? and of course, What exactly triggers expression of genes required for virulence? The research described in this thesis has contributed to a better understanding of the basic biology of the pneumococcus by focusing on (nutritional) regulatory systems that control expression of various proteins that are directly or indirectly involved in virulence. Specifically, we examined the two component system 09 and the transcriptional regulators PsaR, CodY, and GlnR.

Two-component signal transduction systems (TCSs) play an important role in pneumococcal physiology by regulating various cellular processes (22, 33). They sense environmental stimuli that, when present, activate a signaling cascade. As was previously shown by Blue and Mitchell, response regulator 09 (*rr09*) has a strain-specific role in virulence in mice: an *rr09* mutant of D39 (serotype 2) was found to be avirulent in all models tested (i.e., colonization, pneumonia, and bacteremia), while an *rr09* mutant in 0100993 (serotype 3) was only attenuated upon intranasal infection (3). We extended these observations to TIGR4 (serotype 4) and showed that TIGR4 RR09 is also required for full virulence upon intranasal infection (**chapter 2** and 15). In addition, we showed that TCS09 controls expression of different sets of genes in D39 and TIGR4. Taken together, these observations suggested that the different virulence properties of these strains might, at least partially, be explained by this differential RR09-regulated gene expression. Especially the operon encoding a putative phosphotransferase system (*sp0060-sp0066*) was found to be differentially expressed between D39, TIGR4, and 0100993. Interestingly, expression of this operon appeared to coincide with the virulence properties of the respective strains (i.e., downregulation of *sp0063* in D39Δ*rr09* and no differential expression in TIGR4Δ*rr09*). However, a D39Δ*sp0063* mutant was not attenuated in a murine model of infection, indicating

that the observed virulence phenotype of the *rr09* mutant was not caused by the downregulation of this gene.

Another interesting finding was the RR09-dependent regulation of the *rlrA* pathogenicity islet (encoding a regulator, a structural pilus, and 3 sortases) (2). In TIGR4Δ*rr09*, expression of this islet was downregulated, indicating direct or indirect regulation by RR09. In a recent paper, TCS08 has been shown to regulate the expression of the *rlrA* pathogenicity islet as well (31). In this paper, Song and co-workers found that in TIGRΔ*rr08* expression of the *rlrA* pathogenicity islet was increased in late stationary phase, which is in agreement with our findings (15). In another recent paper, Rosch and co-workers showed that the regulation of the *rlrA* pathogenicity islet is even more complex, with two other TCSs, and the transcriptional regulators MerR (or CzcD) and PsaR controlling the expression of the *rlrA* pathogenicity islet as well (27). As described in chapter 3, we confirmed regulation of expression of the *rlrA* pathogenicity islet in TIGR4 (13). In addition, MerR (*sp1856*) was also differentially expressed in the TIGR4 *psaR* mutant (13). Interestingly, it seems that multiple signals, e.g., manganese concentrations for PsaR and unknown for RR09, control the expression of this islet. Rosch and co-workers conclude that this pathogenicity islet (acquired by genetic recombination) integrated into the preexisting regulatory networks of pneumococcal strains, and that this integration might have occurred differently in the various strains (or serotypes). It will be interesting to see how TCSs and especially TCS09, regulate gene expression in other strains than the ones tested in **chapter 2**. In addition, it is as yet unknown whether the identified target genes are directly or indirectly regulated by RR09, or to which external signal the histidine kinase sensor protein responds. Sequencing of several different strains (or serotypes) and testing global gene expression might give some insight into the plasticity of pneumococcal two-component systems.

The role of metallic cations during infection is a well-studied area of microbial pathogenesis, as is the competition between host and pathogen for these ions (29). During colonization and infection of humans, pneumococcus is exposed to fluctuating concentrations of cations, making strict regulation of their uptake of utmost importance. For example, manganese (Mn^{2+}) is essential to all bacteria, because it serves as a cofactor for several metalloenzymes (16). Fluctuation of Mn^{2+} concentrations might serve as a trigger for site-specific expression of virulence factors, since the concentration of Mn^{2+} is much higher in saliva than in blood plasma (6). Like Mn^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+} have been described to be important to bacterial virulence (7, 9, 10, 30, 34). The pneumococcal Mn^{2+}-regulator PsaR (17, 19) was investigated in **chapter 3** (13). Previous studies showed that PsaR is important

for virulence in a serotype 3 strain (11), and we extended these studies using two other strains, D39 (serotype 2) and TIGR4 (serotype 4). Mutants lacking *psaR* displayed strain-specific phenotypes, evident mainly from differences in its contribution to development of bacteremia in mice. In addition, we also observed genes to be affected in expression in the *psaR* mutant in only one of the tested strain backgrounds. Among these strain-specific regulated genes was the *rlrA* pathogenicity islet (see above) (27). The *psa* operon, *prtA*, and *pcpA* were confirmed as PsaR-targets in both D39 and TIGR4 in our study (17, 19), One of these common PsaR targets, the choline-binding protein PcpA, was demonstrated to be required for adherence to a human pharyngeal cell line (12, 28). Moreover, regulation of expression of this adherence factor was shown to be directly positively affected by the nutritional regulator CodY (12 and **chapter 4**), while PsaR negatively regulates the expression of *pcpA*. CodY was shown to bind to the *pcpA* promoter regions thereby activating transcription of *pcpA* (12). This suggests a direct mechanism for adherence of pneumococcal cells to pharyngeal cells as a reaction on nutrient availability (**chapter 7**). These results are underscored by several studies showing higher expression of *psaA* and *pcpA* in *in vivo* situations (e.g., during contact with nasopharynx epithelial cells, and in the murine nose, lungs and blood) (20, 21). Contradictory results were also described: *pcpA* is not involved in colonization, but in invasive disease (8, Cron *et al.*, personal communication). In addition, Bootsma *et al.* found that expression of *pcpA* was lower in nasopharyngeal lavage fluid than in culture medium (Bootsma *et al.*, personal communication, see also **chapter 7**). Thus, the exact role of PcpA during pneumococcal disease remains unclear. The data described above imply that strain-specificity of pneumococcus may complicate the examination of the cellular processes involved in virulence. Moreover, it suggests that expression of potential pneumococcal antigens may occur in a strain-specific manner, similar to the variety of capsular serotypes.

Nitrogen is a vital element for virtually all biological molecules. Therefore, bacteria have to ensure that they can utilize nitrogen from their continuously changing environment. Bacteria have developed sophisticated uptake and metabolic systems for the acquisition of nitrogen-containing molecules. In several Gram-positive bacteria, these systems have been well studied, but not much was known about the importance of nitrogen regulatory systems during the pneumococcal life cycle. In **chapters 4, 5, and 6** we investigated the two main regulatory systems controlling nitrogen uptake, metabolism and inter-conversion in *S. pneumonia*, CodY and GlnR (12, 14, 18). Since we already extensively described these regulatory systems in **Chapter 7**, they will only be discussed briefly here.

CodY was found to be required for adherence and colonization (12 and **chapter 4**). Many transport systems involved in nitrogen uptake and metabolism were found to be regulated by CodY. A changed expression of one of these genes (or a combination of these) might be responsible for the lack-of-colonization phenotype and the lower adherence of the *codY* mutant. The *pcpA* gene was more highly expressed in the *codY* mutant, however, as described above, it seems not very likely that this gene is required for colonization and adherence.

The transcriptional regulator GlnR was investigated in **chapters 5 and 6**. This glutamine/glutamate-dependent regulator itself was found not to be essential for pneumococcal virulence in mice, but its target genes were: glutamine synthetase GlnA was required for efficient colonization and the glutamine permease GlnP was required for survival in the lungs (14). A double mutant for *glnA* and *glnP* appeared to be avirulent in all our models. Interestingly, microarray analysis showed that in this double mutant many CodY targets were overexpressed, indicating that the double mutant was in severe nutritional stress. Our results clearly show that both the CodY and GlnR regulatory systems are important for pneumococcal virulence. Given the strain-specificity observed by us and others for other regulatory systems, it will be interesting to see if these two highly conserved regulatory proteins also have strain-specific features.

Concluding remarks

In this thesis four different regulatory systems have been investigated. In different ways, all were shown to be involved in virulence. A major finding in this thesis is that the target genes of several conserved regulators appear to be strain-specific (**chapters 2** and **3**), which is rather surprising considering that these regulatory systems are conserved among the sequenced strains and even among other streptococci. Strain-specificity complicates the discovery of new antigens that could serve as novel vaccine targets. Besides strain-specific regulation of gene expression, genes are also expressed site-specifically, e.g., expressed at the nasopharynx but not in the blood. One can argue that for vaccination purposes, the ideal situation would be expression of an antigen only during invasive disease and not during colonization; this will leave colonization unaffected and will prevent invasive disease. The benefit of leaving colonization unaffected is that it keeps other potentially pathogenic bacteria from colonizing due to competition between pneumococcus and other respiratory pathogens (4, 23).

Another important finding described in this thesis is requirement of the two nutritional regulators CodY and GlnR for full pneumococcal virulence (**chapter 4, 5, and 6**). Obviously, nutrition is the most important process in (bacterial) survival; without food no living organism can survive. Under normal conditions, pneumococcus only inhabits the nasopharynx of humans. This highly specific niche probably has a highly specific pool of metabolizable nutrients for which pneumococcus has developed specific metabolic routes. This makes it very likely that expression (or repression) of genes involved in nutrient uptake/breakdown and colonization are linked and co-regulated. **Chapters 4**, **5**, and **6** have contributed to a general understanding of this gene regulation. Future research should be focusing on elucidation of these regulatory pathways to get a better understanding of pneumococcal behavior. For instance, CodY is repressing its gene targets during colonization (12), but it remains to be investigated whether CodY-regulated genes are required for invasive disease. It will be interesting to see the virulence phenotype of a CodY-overexpressing pneumococcus during a pneumonia or bacteremia model of infection.

In summary, this thesis highlights regulatory networks controlling the interplay between pneumococcal nutrition and virulence. Unraveling the gene transcriptional networks will help predict bacterial behavior and, consequently, will aid future disease prevention and treatment strategies.

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Samenvatting en discussie

Streptococcus pneumoniae, beter bekend als de pneumokok, is een belangrijke verwekker van verschillende infectieziekten bij mensen. Deze bacterie koloniseert de nasopharynx, een specifiek deel in de neus-keel holte (waar de neus overgaat in de keel), zonder de gastheer ziek te maken. In gezonde mensen verloopt dragerschap asymptomatisch, maar in mensen met een verzwakt immuunsysteem kan het leiden tot ernstige infecties, zoals oor-, long-, en hersenvlies-ontsteking, maar ook infectie van het bloed. Het huidige pneumokokkenvaccin is gericht tegen het suikerkapsel en beschermt goed tegen de meeste pneumokokkeninfecties. Dit komt doordat het vaccin kolonisatie voorkomt. Kolonisatie is proces dat voorafgaat aan zo goed als alle andere pneumokokkeninfecties (zoals oor-, long- en hersenvliesonsteking). Problemen hierbij zijn dat de pneumokok verschillende verschijningsvormen heeft, de zogenaamde serotypen. Het vaccin beschermt maar tegen 7 van de meer dan 90 bekende serotypen. Het serotype wordt bepaald door de structuur van het suikerkapsel, dat rondom de pneumokok zit. Dit suikerkapsel geeft de bacterie bescherming tegen het immuunsysteem en kan zich zodoende handhaven in het menselijk lichaam. Huidig onderzoek richt zich vooral op de eiwitten die aan het oppervlakte van de bacterie zitten. De reden hiervoor is dat deze eiwitten door het menselijk immuunsysteem herkend kunnen worden. Na herkenning kan de bacterie vervolgens onschadelijk gemaakt worden door het immuunsysteem met behulp van o.a. antistoffen. Veel van deze eiwitten zijn al getest op het vermogen tot antilichaamproductie door het menselijk lichaam en sommige hiervan blijken inderdaad een beschermende werking tegen pneumokokkeninfectie te hebben. Van veel van deze eiwitten is het niet bekend wanneer en waar in het menselijk lichaam ze tot expressie komen. Met andere woorden, wanneer zitten die eiwitten aan het oppervlakte van de bacterie en dus wanneer zijn ze bereikbaar voor het immuunsysteem? Dit proefschrift beschrijft vier intracellulaire regulatie systemen die de expressie van verschillende eiwitten reguleren: het "two-component system 09", en de transcriptionele regulators PsaR, CodY en GlnR.

De pneumokok heeft dertien zogenaamde "two-component signal transduction systems" (TCS), signaal transductie systemen bestaande uit twee eiwitten: een sensor eiwit (histidine kinase) en een DNA-bindende response regulator. Het sensor eiwit bevindt zich in de cel membraan en "tast" de omgeving van de bacterie af voor bepaalde signalen. Deze signalen kunnen voedingstoffen zijn, maar ook bijvoorbeeld pH en osmotische waarde van de omgeving. Het DNA-bindende eiwit bevindt zich in de cel waar het promotergebieden van genen op het DNA kan binden. Deze promotergebieden dienen als herkenningsplek op het DNA voor de response regulator. Na binding van de response regulator aan de promoter,

kunnen genen aangezet of uitgezet worden. Het TCS systeem dat wij bekeken hebben is TCS09 waarvan de functie nog onbekend is (**hoofdstuk 2**). Van TCS09 hebben wij het gen dat codeert voor de response regulator (*rr09*) uitgeschakeld. Vervolgens hebben we met behulp van infectiemodellen in muizen gekeken naar de ziekmakendheid (virulentie) van deze mutante pneumokokken. Uit een studie uit 2003, door Blue en Mitchell, bleek dat na uitschakeling van *rr09* in twee verschillende pneumokokkenstammen, D39 en 0100993 (ook verschillend in serotype), een verschillende uitwerking hadden op de virulentie van de bacteriën; een mutant voor *rr09* in D39 was helemaal niet meer virulent, terwijl de 0100993 *rr09* mutant nog wel ziekte kon veroorzaken. Wij hebben ook een derde serotype onderzocht, genaamd TIGR4. Deze bleek nog virulent, maar wel wat minder dan de 0100993. Om een idee te krijgen over de functie van TCS09 in de pneumokok, hebben we de expressie van alle genen in de D39 en TIGR4 *rr09* mutanten vergeleken met die van de wild-type bacterie met behulp van microarrays. Met deze technologie kun je in één experiment naar de expressie van elk gen van de bacterie kijken. Hieruit bleken heel veel genen een veranderde expressie te hebben door de mutatie van *rr09*. Van een aantal van deze genen is de expressie bekeken in bacteriën geïsoleerd uit muizen. Dit was destijds een relatief nieuwe techniek, hiermee hebben we aan kunnen tonen dat die verschillende genen zich ook anders gedroegen in een muis. Helaas kon dit niet voor de D39 *rr09* mutant, aangezien die zich niet kon handhaven in de muis. Een gen, nog zonder naam, geclassificeerd als *sp0063*, bleek in de D39 *rr09* mutant (gegroeid in een medium en dus niet in een muis), een erg lage expressie te hebben in vergelijk met de D39 wild-type. Interessant genoeg bleek dit niet het geval voor de TIGR4 *rr09* mutant en voor de 0100993 *rr09* mutant. De expressie van dit gen kwam overeen met het patroon van virulentie in de dierproeven. Als gevolg van deze observatie hebben we ook een mutant voor *sp0063* gemaakt en deze in een infectiemodel getest op virulentie. Deze *sp0063* mutant bleek uiteindelijk ook virulent te zijn. Hierdoor moesten we concluderen dat het gebrek van expressie van dat gen niet verantwoordelijk kan zijn voor het avirulente gedrag van de D39 *rr09* mutant.

Een ander interessante bevinding was dat RR09 het "*rlrA* pathogenicity islet" reguleert in de TIGR4 stam. Dit *rlrA* pathogenicity islet bevat een aantal genen die coderen voor een pilus. Een pilus is een haarachtige structuur die, in dit geval, betrokken is bij hechting van bacteriën aan oppervlakten, bijvoorbeeld een menselijke cel. In de TIGR4 *rr09* mutant bleek de expressie van de pilus genen sterk te zijn afgenomen. In een artikel door Song en collega's, wordt beschreven dat deze genen ook gereguleerd worden door TCS08. In een derde artikel door Rosch en collega's, wordt beschreven dat er nog meer regulatoren het *rlrA* islet

reguleren namelijk, twee two-component systemen en twee transcriptionele regulatoren genaamd MerR (of CzcD) en PsaR. Een transcriptionele regulator is, vergelijkbaar met de response regulator, een eiwit dat DNA kan binden op een herkenningsplek specifiek voor de regulator. Eenmaal gebonden kan het genen aan- of uitzetten. Het lijkt er dus op dat deze genen beïnvloed worden door meerdere signalen: divalente kation concentraties voor MerR en PsaR (zie onder), en een nog onbekend signaal voor RR09. Een artikel door Rosch beschrijft dat het *rlrA* pathogenicity islet is opgenomen door de pneumokok uit zijn directe omgeving. De locatie van het stuk DNA op het chromosoom is niet in elke stam hetzelfde. Hierdoor is de regulatie van het *rlrA* pathogenicity islet niet hetzelfde in de pneumokokkenstammen. Het is vooralsnog niet bekend of de genen die wij geïdentificeerd hebben ook direct gereguleerd worden door RR09. Bovendien is ook het externe signaal, waar TCS09 op reageert, niet bekend. Door de verschillende pneumokokkenstammen te onderzoeken op het gebied van TCS09 en de regulatie van het *rlrA* pathogenicity islet, zal blijken hoe flexibel het pneumokokkengenoom en de regulatie door two-component systemen zijn.

Mangaan (ook wel Mn^{2+}) is een belangrijk metaal-ion, omdat het nodig is als cofactor voor de activiteit van vele eiwitten. In de pneumokok wordt de opname van dit ion o.a. gereguleerd door de transcriptionele regulator PsaR (**hoofdstuk 3**). Met behulp van pneumokokken waar dit gen was uitgeschakeld, hebben we gekeken naar de bijdrage van deze regulator aan virulentie in muizen. Hieruit bleek dat PsaR nodig is om vergelijkbaar virulent in de bloedbaan te zijn als de wild-type pneumokok. Ook zagen we weer dat er verschillen waren tussen de onderzochte stammen D39 en TIGR4. Net als voor RR09 (**hoofdstuk 2**), hebben we gekeken naar de expressie van PsaR-gereguleerde genen m.b.v. microarrays. Hieruit kwam dat die genen, die al eerder in de literatuur beschreven waren als zijnde gereguleerd door PsaR, inderdaad ook een andere expressie hadden in onze *psaR* mutanten. Bovendien zagen we een set andere genen met een veranderde expressie die verschilde tussen D39 en TIGR4. Met behulp van een tweede techniek (die de hoeveelheid eiwitten van de cel vergelijkt) hebben we voor de direct gereguleerde genen, de *psa* genen, *prtA* en *pcpA*, maar ook een aantal andere genen de resultaten kunnen bevestigen. Een van de direct door PsaR gereguleerde genen codeert voor het PcpA eiwit. In **hoofdstuk 4** hebben we laten zien dat de expressie van *pcpA* wordt gestimuleerd door CodY, een transciptionele regulator die reageert op het voedselaanbod van de bacterie. Ook hebben we laten zien dat PcpA nodig is voor het plakken aan een cellijn van menselijke nasopharynxcellen. Dus de regulatie van *pcpA* door CodY als reactie op het voedselaanbod lijkt een direct moleculair mechanisme te zijn.

Bevindingen die dit onderbouwen, weliswaar indirect, zijn beschreven in de literatuur. Echter, tegenstrijdige bevindingen zijn er ook; een *pcpA* mutant koloniseert de nasopharynx van de muis vergelijkbaar met de wild-type pneumokok (Cron *et al.*, persoonlijke communicatie). Dus de exacte rol die PcpA in het infectieproces speelt is nog verre van duidelijk.

Stikstof is een element dat voorkomt in bijna elk biologisch relevant molecuul. Aminozuren zijn zulke stikstof houdende bouwstenen en vormen onder andere eiwitten en andere celcomponenten. Hierdoor zijn ze absoluut onmisbaar voor de cel. Kortom zonder stikstof en aminozuren geen leven. CodY is een regulatoreiwit betrokken bij de regulatie van genen die verantwoordelijk zijn voor de opname en de omzetting van stikstofhoudende moleculen en aminozuren. In andere, aan de pneumokok verwante, bacteriën is dit eiwit al uitgebreid onderzocht en daar uit bleek dat CodY genen uitzet als er voldoende stikstof en aminozuren zijn. Als er te weinig nutriënten zijn, zal CodY deze inactivatie stoppen, waardoor de genen dus aangezet worden. Wij hebben de rol van CodY in stikstof en aminozuur metabolisme in relatie tot virulentie in de pneumokok onderzocht (**hoofdstuk 4**). Hieruit bleek dat een pneumokok, waar het *codY* gen is uitgeschakeld, zich niet meer zo goed kan handhaven in de neus van een muis. Ook bleek dat deze mutant minder goed aan een cellijn van menselijke nasopharynx cellen kon plakken (adhereren). Met andere woorden, CodY is belangrijk voor de eerste fases van het infectieproces, de kolonisatie en adherentie. In de *codY* mutant, in cultuurmedium gegroeid, bleken ook veel genen anders tot expressie te komen dan in een niet-mutante bacterie: veel genen coderend voor eiwitten, die betrokken zijn bij de opname van stikstof-houdende nutrienten en aminozuren en de intracellulaire omzetting van deze nutrienten, waren meer actief. Eén of een combinatie van deze genen zou dus een direct effect kunnen op de kolonisatie en adherentie kunnen hebben. Het gen *pcpA* kwam hoger tot expressie bij deze genen, maar is dus zeer waarschijnlijk niet direct betrokken bij kolonisatie en adherentie (zie boven).

Een tweede stikstof en aminozuur regulatiesysteem is onderzocht en beschreven in de **hoofdstukken 5** en **6**. GlnR is een eiwit dat de expressie van genen betrokken bij het glutamaat en glutamine metabolisme reguleert. Glutamaat en glutamine zijn zeer belangrijke aminozuren, niet in de eerste plaats omdat ze bouwstenen zijn van eiwitten, maar ze zijn ook nodig om moleculen zoals DNA, de celwand en het suikerkapsel van te maken. Het bleek dat de volgende genen gereguleerd worden door GlnR: *glnA*, een eiwit dat van glutamaat glutamine maakt; *glnPQ*, een glutamine/glutamaat opname systeem; en *gdhA*, een eiwit dat glutamaat maakt uit andere moleculen (**hoofdstuk 5**). Om de rol van deze genen in virulentie te bestuderen, hebben we mutanten voor GlnR-gereguleerde genen getest in infectiemodellen

met behulp van muizen. Ook is er naar de adhesie aan menselijke nasopharynx cellen gekeken (**hoofdstuk 6**). Hieruit bleek dat met name GlnA en GlnP belangrijk zijn voor de virulentie van de pneumokok. GlnA is nodig voor het plakken aan menselijke nasopharynx cellen, een goede kolonisatie van de muizennasopharynx, en voor het overleven in de bloedbaan van muizen. GlnP bleek nodig voor het overleven van de pneumokok in de longen van de muis en waarschijnlijk ook voor de overgang van de longen naar de bloedbaan. Uit deze resultaten bleek dat verschillende genen van het zelfde regulatie systeem nodig zijn op verschillende momenten en locaties tijdens infectie van muizen. De regulatie systemen die stikstof en aminozuur metabolisme reguleren zijn dus erg belangrijk voor het overleven van de pneumokok tijdens zijn verblijf in de gastheer. Uit de literatuur blijkt dat ook andere cellulaire regulatie systemen gekoppeld zijn aan CodY en GlnR (**hoofdstuk 7)** en deze zijn ook belangrijk voor de virulentie en het overleven van de bacterie.

Tot slot, dit proefschrift levert een wetenschappelijke bijdrage aan het pneumokokkenonderzoek, met als belangrijkste bevinding dat regulatie van virulentie en metabolisme aan elkaar gekoppeld zijn. Het in kaart brengen van alle regulatiesystemen van de pneumokok zal tot een beter begrip van de bacterie leiden met als gevolg een verbeterde preventie en behandeling van pneumokok-gerelateerde ziekten.

Curriculum Vitae

Wouter Hendriksen was born in Stad Delden on the 27th of February 1978. With his parents Theo and Ineke and older brother Bas, he moved to Almelo at age four. After primary school, he went to the Pius X College in Almelo, where he obtained first his HAVO and then his VWO degree.

In 1997 he started studying Biology at the Rijksuniversiteit Groningen (RuG) in Haren. During this study, he specialized in Molecular Biology, finishing an internship in the group Molecular Microbiology (RuG) headed by Professor Arnold Driessen. The subject of this internship was ABC-transporters in *Penicillium chrysogenum*. His second internship was undertaken in Evanston, Illinois in the United States of America. Here, at the Chemical Engineering department of the Northwestern University, he investigated the transcriptional regulator Spo0A of *Clostridium acetobutylicum* under supervision of Dr. Hendrik Bonarius and Professor E. Terry Papoutsakis. After these internships, he decided to write his "doctoraal" thesis under supervision of Professor Oscar Kuipers, head of the Molecular Genetics department (RuG).

After earning his MSc degree, he performed his PhD studies under supervision of Professor Peter Hermans and Professor Ronald de Groot. During this time he was a member of the Molecular Medicine PhD committee for three years. At present, Wouter is Postdoc at the Institute of Biology, Leiden University, in the group of Dr. Paul van Heusden, where he studies the role of 14-3-3 proteins in cation homeostasis in the yeast *Saccharomyces cerevisiae*.

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