Regulatory responses of Streptococcus pneumoniae to varying metal ion- and nitrogen availability
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Chapter 1

Introduction
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Bacteria are able to survive on almost all places on earth, sometimes under extremely harsh conditions, like hot pools, hyper-osmotic seawater and even inside other living organisms. When bacteria are able to inhabit (parts of) the human body and are able to cause disease, i.e. harm the well functioning of the body, they are called human pathogens. Some human pathogens enter the body via food or water, like *Salmonella* species and certain *Bacillus* species, while others permanently inhabit the human population and are spread horizontally through (close) contact between people.

An example of a pathogen belonging to this latter class is *Streptococcus pneumoniae*, also known as pneumococcus (133,176,317), a Gram-positive bacterium with a relatively small genome size of around 2200 genes. It belongs to the genus of streptococci, among which several other important human and animal pathogens, like *Streptococcus pyogenes* (group B *Streptococcus*), *Streptococcus agalactiae* (group A *Streptococcus*), *Streptococcus sanguinis*, *Streptococcus suis*, *Streptococcus uberis*, *Streptococcus equi*, *Streptococcus mutans*, and is closely related to the lactic acid bacteria *Lactococcus lactis* and *Streptococcus thermophilus*.

*S. pneumoniae* was the first bacterium shown to be competent for genetic transformation, i.e. able to take up genetic material from the environment, and based on this characteristic it was used to demonstrate that DNA contains heritable traits (19,101). Nowadays, pneumococcus is still a model organism for the study of the molecular biology of competence (63), but besides that, a huge effort has been carried out concerning research into the pathogenic properties of the bacterium (151). Moreover, with the availability of the genome sequences of various pneumococcal strains, the number of molecular and epidemiological studies on *S. pneumoniae* has expanded exponentially.

This introduction deals with several topics with respect to *S. pneumoniae*, including important virulence factors it possesses, types of disease it may cause, treatment and prevention of pneumococcal diseases, (genome-wide) methods that have recently been developed to study the organism on the molecular level, and the importance of transcriptional gene regulation for the bacterium’s adaptation to and survival in the continuously changing environment in the human body. The experimental work described in this thesis has made a significant contribution to these last two points. First, it presents the development and application of a technique for genome-wide negative screening of conditionally essential genes. Second, it provides novel findings on the interplay of *S. pneumoniae* with its surrounding milieu by having investigated the mechanisms of transcriptional gene regulation in response to two important extracellular stimuli, namely the nitrogen source and divalent cations.

**S. pneumoniae: diseases, virulence factors and therapies**

**Diseases**

The pneumococcus is an opportunistic pathogen that resides as a commensal in the nasopharynx (Fig. 1) of a large fraction of the human population (see 40,151,217,235 for reviews). Asymptomatic nasopharyngeal pneumococcal carriage is usually the highest in children, and epidemiological studies have found the percentage of carriage to be as high as a few- to over 80% depending on factors like age, geographical origin, exposure to crowding and coincidence with other diseases like HIV or sickle-cell disease (reviewed in 40). Asymptomatic carriage is believed to be an important source of horizontal spread of pneumococcal strains, which especially occurs in children because of their higher frequency of nasopharyngeal colonization (40). Following carriage, the bacterium may spread in susceptible people to several other sites of the body, such as the lungs, middle ears, sinuses,
blood stream and meninges, giving rise to diseases like pneumonia, otitis media (=middle ear infection), sinusitis, sepsis and meningitis (Fig. 1). Although a large percentage of people is carrier of \textit{S. pneumoniae}, carriership usually does not result in disease. However, young children, with a still developing immune system, as well as elderly people and individuals with immunodeficiencies are specific risk groups for getting disease (40,151).

Figure 1. Diseases caused by \textit{S. pneumoniae}. Black ovals indicate diseases that establish via the bloodstream, and grey ovals represent spots of diseases that can be acquired via the airways.

Worldwide, pneumococcus is estimated to be responsible for more than 1 million of deaths annually caused by pneumonia, meningitis, sepsis and other diseases in children (151,235). In the USA pneumococcal infection causes 40,000 fatal cases yearly, and 30-60% of the survivors develop long term effects such as neurological disorders (40). In addition, \textit{S. pneumoniae} has a high impact as a cause of morbidity, since for example pneumococcal pneumonia may occur in over 1% of the human population in all age groups (235). Hence, there is a huge effort to identify and understand the function of factors enabling \textit{S. pneumoniae} to effectively survive in the body, \textit{i.e.} factors that contribute to the virulence - the degree of pathogenicity or the relative ability to cause disease - of the organism, so-called virulence factors. Nowadays, several virulence factors are known, that in various ways and to variable degrees contribute to the overall fitness and virulence of \textit{S. pneumoniae} in different sites of the human body. A number of these have potential as vaccine targets.

\textbf{Virulence factors}

The pneumococcus possesses a large number of genes, mutation of which has been shown by \textit{in vivo} infection models to attenuate virulence (142,143). The most important and well-known virulence factor is the capsule polysaccharide (337). This is a layer of carbohydrate polymers surrounding the cell-wall of the pneumococcus and it is highly variable, regarding the 90 different pneumococcal serotypes, each with a specific type of capsule (142,329). The capsule protects the bacterium against phagocytosis (complement-mediated opsonophagocytosis), trapping in neutrophil extracellular traps (NETs, 336), extracellular webs that the host cells employ to kill pathogens, and it plays a key role in
systemic dissemination of the pneumococcus after invasion (46,196). On the other hand, it impairs efficient adhesion to respiratory epithelial cells-layers, which is necessary to colonize the nasopharynx (3,68,339). In connection with this, Hammerschmidt et al. have shown by electron microscopy that bacteria in intimate contact with epithelial cells have a thinner capsule layer, indicating that they may downregulate capsule expression in this specific situation in order to enhance adherence (114).

Another well-known virulence factor is the pneumococcal cytolysin pneumolysin (130). Pneumolysin kills host cells by creating pores in the membrane using cholesterol as a receptor, in this way creating tissue damage (130). Many studies have also shown an important contribution of pneumolysin to invasive pneumococcal disease (130). Other consequences of pneumolysin action are induction of complement activation (273) and proinflammatory reactions in immune cells (64).

Since the outside of the cell stays in direct contact with the extracellular milieu, obviously a lot of important pneumococcal virulence factors are proteins displayed on the cell surface. Well-studied peptidoglycan-bound virulence factors are the neuraminidases NanA and NanB, which cleave host sialic acid from host surface glycans, like mucin, glycolipids and glycoproteins, which may promote colonization (33,142,291,323), and HylA, a hyaluronate lyase that degrades hyaluronan, a component of the extracellular matrix of host tissues (194). There are also cell wall sorted extracellular Zn\(^{2+}\) metalloproteinases (Zmp) implicated in virulence, where ZmpC was found to cleave human metalloproteinase 9 and to be involved in pathogenicity in the lung, and ZmpB in induction of inflammation in the lower respiratory tract (39,59,237). Another important virulence factor built from cell wall sorted proteins in pneumococci is the pilus, encoded by the \textit{rlrA} pathogenicity islet which affects virulence and adhesion, and evokes host inflammatory responses (21,191).

The pneumococcal cell wall contains the unusual constituent choline, which serves amongst others as an anchor for a group of surface proteins called choline binding proteins, of which there are 15 encoded by the genome of strain TIGR4 (30). These bind in a noncovalent manner to choline moieties in the techoic and lipoteichoic acids and are known to be important for virulence (100,272). To several choline binding proteins a role in adhesion has been attributed, such as the well-studied CbpA, which is a surface adhesin (201) and the choline binding protein PcpA (127). Another group of choline binding proteins is formed by the murein hydrolases LytA, B and C, involved in processes such as cell separation and cell wall breakdown during competence, resulting in lysis and DNA release (72,107,269). The choline binding protein called “pneumococcal surface protein A” (PspA) interferes with complement deposition, thereby reducing clearance of pneumococcus by the complement receptor mediated pathway (262,350). In addition, PspA is known to bind human lactoferrin and may in this way contribute to iron acquisition at mucosal surfaces (113).

A third class of surface proteins that include virulence factors are the lipoproteins. There are over 40 lipoproteins encoded by the \textit{S. pneumoniae} genome (30,112). A well-studied example is the pneumococcal surface antigen A (PsaA), which is a Mn\(^{2+}\) uptake ABC transporter, that is also needed for proper adhesion (11,79,326).

Last, several proteins lacking one of the mentioned surface binding characteristics are known, that nonetheless appear at the pneumococcal surface. For instance, the glycolytic enzymes α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have affinity for plasminogen, contribute to virulence and are displayed on the cell surface (31,32,85). Another sugar metabolic enzyme, 6-phosphogluconate dehydrogenase, is likewise displayed at the surface of \textit{S. pneumoniae}, where it acts as adhesin/lectin and has immunogenic properties (70). Also for PavA, pneumococcal adherence and virulence protein A, a
fibronectin binding protein involved in virulence, adherence, invasion and meningeal inflammation, it is unknown how it ends up on the cell surface (131,257).

The virulence factors described above mostly appear to have specific functions during interaction with the host. However, a lot of studies have also reported smaller or larger effects on virulence of mutations in genes that encode proteins involved in cellular processes that do not have an obvious direct link with virulence, for example metabolic pathways that are present in nonpathogenic bacteria as well. Although one could argue that these genes do not encode true virulence factors, it is interesting to see that some proteins encoded by "housekeeping" genes have a large impact on pneumococcal virulence, which is sometimes site specific, and also might have potential as vaccine targets. Furthermore, some housekeeping genes may have acquired dual functions, like α-enolase, which is, as described above, also involved in plasminogen binding. Examples of this class of virulence factors include gene products involved in metal ion homeostasis, such as the lipoproteins responsible for iron, zinc and manganese uptake (48,49,79,80,211) or oligopeptide uptake Ami-AliA/B (154), the peptidyl-prolyl isomerase lipoprotein SlrA (129), cytosolic enzymes like alkylhydroperoxidase (248), pyruvate oxidase (301), NADH oxidase (18,349) the endonuclease EndA, which is involved in release of pneumococci from NETs as well as in genetic competence (25), sortase A (SrtA) required for anchoring of LPXTG containing proteins to the pneumococcal cell wall (58,249), the heat shock proteins ClpP protease (173) and the nitrogen metabolic enzymes GlnA and GlnP (chapter 7).

Thus, the virulence of \textit{S. pneumoniae} is determined by a diverse set of genes, of which some have a function dedicated to a specific type of interaction with a host factor, whereas others contribute to virulence by increasing fitness in certain tissues in the human body and yet other genes have both a function in general cellular processes, as well as a secondary function in virulence.

**Treatment and prophylaxis**

With the emergence of antibiotic resistant pneumococcal strains (115), there is a high need for an effective vaccine to reduce the incidence of pneumococcus-borne diseases. Until recently, prophylactic strategies used to be based on a 23-valent polysaccharide vaccine directed against the capsule polysaccharide. A disadvantage of this vaccine is, however, that it is quite ineffective for children younger than 2 years (40,41). Therefore, new seven- to eleven-valent polysaccharide vaccines have been developed, which are effective in children younger than two as well (40,41,142). For several reasons, though, current efforts are directed at the development of protein based vaccines: They are likely to be cheaper than existing ones, they are expected to elicit immune responses in all age groups including children younger than 2 years, and they should offer serotype-independent protection, thereby preventing serotype displacement (40).

Several pneumococcal proteins have been evaluated for their potential to elicit an immune response in animal models and protective effects have been found for, amongst others, the choline binding protein PcpA (98), the iron uptake lipoproteins PiuA and PiaA (147,148,340), the polyamine transport protein PotD (290), the heat shock protein ClpP (173), pneumolysin (95), PspA, PsaA (311,312), CbpA (240) and others (96). Interestingly, several studies are going on that explore the use of combinations of pneumococcal virulence proteins as vaccine components and it seems that there is often an additive and even synergistic effect of these cocktails compared to single proteins (239).
Introduction

Molecular tools to study pneumococcus

General classical molecular tools

To be able to dissect *S. pneumoniae* on the molecular/genetic level and to study its interaction with the environment, several useful molecular tools have been developed in recent years, including various gene reporter systems, overexpression systems, plasmid vectors, defined media and methods to make mutants either in a defined way or randomly (1,56,62,86,106,110,161,185,298,310). Recently, the whole spectrum of molecular tools available for the lactic acid bacterium *L. lactis*, which is closely related to *S. pneumoniae*, was adapted for use in the latter, adding several improved and new tools to the existing toolbox (chapter 2). Together with the publication of the genome sequences of several *S. pneumoniae* strains, these methodologies have prompted an explosion of molecular studies on pneumococcus.

Genome-wide (screening) techniques

The availability of the genome sequences of three pneumococcal strains, the unencapsulated laboratory strain R6 (133), its encapsulated parent D39 (176) and the virulent clinical isolate serotype 4 strain TIGR4 (317) has led to the establishment of DNA microarray technology for this pathogen. Microarrays are now being used commonly in the pneumococcal research field to identify the transcriptome under various conditions and to analyze the effects/function of transcriptional regulators (see also chapters 4, 5 and 6, this thesis). Since more and more genomes of *S. pneumoniae* strains will be and have been sequenced, newer generations of microarrays will cover the genomes of several different subspecies.

Several screens have been performed in *S. pneumoniae* in order to identify genes involved in pathogenesis/virulence. Marra and co-workers (207) used differential fluorescence induction, a technique that employs a library of promoter fragments fused to a *gfp* reporter to screen for genes induced by a number of stress conditions, namely iron limitation, temperature shift, elevated CO₂ levels, and models for respiratory tract and otitis media infection. In this way, several genes that are induced under these conditions have been picked up.

Three studies in *S. pneumoniae* aimed at genome-wide identification of genes required for pathogenesis, which was done by negative screening using signature-tagged mutagenesis (STM) (123,181,254), *i.e.* screening for mutant clones lost from a pool of mutants when administered in an animal infection model. This screening method makes use of a transposon with a variable tag to construct mutant libraries, making it possible to discriminate between different mutants in relatively small pools. However, with STM, mutants have to be stored individually and detection of the mutants is cumbersome (17). Furthermore, the saturation of transposon mutagenesis of the library cannot be determined with STM. Indeed, the three STM screens performed in *S. pneumoniae* show little overlap, meaning that these screens were not exhaustive.

Therefore, a microarray-based screening system, named genomic array footprinting (GAF), was recently developed that allows high-throughput negative selection in *S. pneumoniae* (chapter 3). This technique makes use of a transposon with two outward facing T7 RNA polymerase promoters, enabling the specific amplification of transposon insertion sites in a mutant library. In *Salmonella typhimurium* a genome-wide screening method was used similar to GAF, and proven to be suitable for *in vivo* screening for genes required for long term systemic infection of mice (182). Other similar techniques have been described and applied in several other organisms: In *Mycobacterium* and *Bacillus anthracis* a PCR-adapter based approach was used to identify genes essential for growth under various conditions, like
sporulation, germination and growth in different media (71,279,280). Different PCR-based methods were applied to assess the content of essential genes in the \textit{Helicobacter pylori} genome (275) and for genome wide profiling of conditionally essential genes in \textit{Escherichia coli} (283,296). However, PCR-based amplification methods seem to yield data that are less robust and reliable than amplification methods that make use of a T7 promoter, as is the case with GAF (chapter 3). GAF has been employed to identify genes involved in competence development (51), \(\text{Zn}^{2+}\) stress (chapter 3) and is currently being applied to identify genes necessary for optimal virulence/survival in several mouse models of \textit{S. pneumoniae} infection (P.W. Hermans and co-workers, Nijmegen Radboud Medical Centre, University of Nijmegen).

\section*{Role of transcriptional gene regulation: In response to the environment}

\textbf{Environmental conditions encountered by \textit{S. pneumoniae}}

Obviously, pneumococcus is exposed to greatly changing and variable environments during growth in the human body, where the bacterium may be situated in the nasopharynx, tissues of the airways and lungs, bloodstream, meningeal tissues etc. These environmental changes can be differences in nutrients, like sugars and nitrogen sources, micronutrients, temperature, osmotic pressure, pH, oxidative stress and so on. Besides physical variations and fluctuations in nutrient concentrations, \textit{S. pneumoniae} also comes into contact with human (immune) cells and it interacts with several other bacteria in the upper respiratory tract, like \textit{Moraxella catarrhalis}, \textit{H. influenzae}, \textit{N. meningitidis}, \textit{S. aureus} and various haemolytic streptococci (40,291). Detailed knowledge of the effects of environmental conditions on pneumococcal gene expression might aid in developing smarter vaccination strategies. For instance, it might be undesirable to use vaccines that confer protection against both colonization and invasive disease, since this could lead to a shift in the nasopharyngeal flora to increased occupation by other pathogens or pneumococci lacking the selected vaccine antigen (98).

A couple of studies have investigated pneumococcal behaviour in and response to specific environmental conditions, like osmotic stress (47), temperature stress (246), metal ion limitation and -stress (207,225), and chapters 4 and 5, this thesis), oxidative stress (146,211,248,326,347) and acid tolerance (210). A remarkable example of pneumococcal adaptation is its ability to switch between a planktonic lifestyle in the bloodstream and a sessile/biofilm way of life in tissues, which is accompanied by the expression of a specific set of (virulence) genes in each situation (7,238). As discussed below, the regulatory mechanisms behind several of these adaptive responses have been investigated in more detail. A very interesting microarray experiment performed by Orihuela \textit{et al}. aimed at the identification of genes upregulated under \textit{in vivo} conditions, \textit{i.e.} in infected blood of mice, in cerebrospinal fluid of infected rabbits and during attachment of \textit{S. pneumoniae} to pharyngeal epithelial cells (243). The data indicated that these different body compartments elicit site-specific expression patterns of amongst others genes encoding virulence factors, transporters, transcription factors, translation-associated proteins, metabolic enzymes, and genes with unknown function. Interestingly, a few genes, among which the virulence genes \textit{psaA} and \textit{prtA}, of which the expression was analyzed in detail in this thesis (chapter 4), showed elevated expression in all three tested compartments, suggesting that they constitute a core set of virulence genes (243). Another study also identified gene expression changes in \textit{S. pneumoniae} in contact with human lung epithelial cells (299), showing a big response in a serotype 3 virulent strain, but only few effects in the R6 unencapsulated laboratory strain. Interestingly, also the \textit{psaC} \(\text{Mn}^{2+}\) ABC transporter permease gene and the \textit{adcB} \(\text{Zn}^{2+}\) ABC
transporter permease gene were upregulated and mutational analysis showed that the \textit{adcB} gene is indeed involved in adherence to human lung epithelial cells (299).

### Transcriptional regulation in pneumococcus

\textit{S. pneumoniae} is equipped with many one- and two-component transcriptional regulatory systems, which form a main way to accomplish proper and adequate adaptation to the environment, \textit{i.e.} the human host tissues and fluids. Two component systems (TCSs) consist of a response regulator and a sensing component (histidine kinase), which enable the bacterium to interact with the environment (9). The pneumococcal genome contains 13 TCSs and one orphan response regulator, of which 8 play a role in virulence in a respiratory tract infection model (320). Several virulence genes are regulated by TCSs. TCS06 regulates expression of \textit{cbpA (pspC)} a major pneumococcal virulence factor, protective antigen and adhesin (202,305,306). In strain D39 it also regulates the virulence gene \textit{pspA} (305,306). \textit{PspA} is regulated by the \textit{YycFG (VicRK)} TCS as well (227). \textit{YycFG} is an essential TCS, that modulates expression of fatty acid biosynthesis genes and is important for cell wall synthesis (226). Expression of the virulence genes \textit{psaBCA} is regulated by TCS04 in strain TIGR4 but not in D39 (212). Such a strain-dependent role has also been seen for TCS09, involved in regulation of various genes (38,128). \textit{HtrA}, encoding an extracellular serine protease and major pneumococcal virulence factor, is regulated by the \textit{CiaRH} TCS (289). Next to \textit{htrA}, \textit{CiaRH} controls several other genes/processes, amongst which 5 genes encoding small non-coding RNAs, of which the function is not yet understood (111). Other pneumococcal TCSs are involved in bacteriocin production (BlpRH) (75), competence (83,105); ComDE, MicAB) and cellobiose metabolism (TCS08) (213). The orphan response regulator \textit{RitR} functions as a regulator of iron transport (328).

STM screens have found 20 one-component regulators that are necessary for full pneumococcal virulence (125). Some regulators affect virulence by controlling the expression of virulence genes. For example, \textit{RegR} and \textit{RegM} regulate expression of the hyaluronidase gene and the capsule biosynthesis locus, respectively, and are important for virulence (57,97). Furthermore, the \textit{rlr} pathogenicity island, encoding a pneumococcal pilus is controlled by \textit{RlrA} and \textit{MgrA} (124,126). There are also regulators of general metabolic pathways that influence virulence. For example, the nutritional regulator \textit{CodY} is a transcriptional repressor of various amino acid metabolic genes, and is necessary for efficient colonization of the nasopharynx (127). Other transcriptional regulators that have been characterized to date in \textit{S. pneumoniae}, some of which have been shown to contribute to virulence as well, include the glutamine regulator \textit{GlnR} (chapter 6), the serine/threonine protein kinase \textit{StkP} that functions as a global controller of gene expression (278), the metal responsive regulators \textit{PsAR} (145) and \textit{ScZA} (chapter 5), the pyruvate oxidase regulatory gene \textit{SpXR} (261) and the carbon catabolite control protein \textit{CcpA} (138,153). Although the gene targets of a lot of transcriptional regulators are known, for many the specific factors to which they respond remain to be identified.

By using bioinformatics and microarray analyses, in combination with classical molecular techniques, the functions of many transcriptional regulators in \textit{S. pneumoniae} with yet unknown functions are currently being characterized. Two pneumococcal regulatory processes will be addressed in this thesis: \textit{i)} the transcriptional response to the extracellular \textit{Zn$^{2+}$} concentration, and \textit{ii)} the regulation of nitrogen metabolism, focussing on glutamine/glutamate metabolism.

### Regulation of the metabolism of trace metal ions

A main topic in this thesis is the regulation in response to and metabolism of trace metal ions, in particular \textit{Zn$^{2+}$} (Fig. 2). When discussing metal ion homeostasis in relation to
pathogens, a classical example is the competition between the host and the pathogen for iron. The pathogen secretes iron-binding siderophores, while the host tries to sequester iron in the form of complexes with hemoproteins and ferritin, thereby rendering the concentration of free iron as low as $10^{-24}$ M (215). However, not only iron, but also other metal ions, such as Zn$^{2+}$ and Mn$^{2+}$, fulfill important functions in the cell, like being co-factors for enzymes or assisting in redox reactions (118,120). For the human host on the other hand, adequate concentrations of trace metal ions, especially Zn$^{2+}$ and Cu$^{2+}$, have been shown to be essential for the proper functioning of the immune system (42,88,136,266,292). Serum levels of Zn$^{2+}$, Mn$^{2+}$ and Cu$^{2+}$ are approximately 25 $\mu$M, 9 nM and 15 $\mu$M, respectively (335). Both Zn$^{2+}$ and Mn$^{2+}$ levels might however be highly different in other tissues, for example in lung tissue, which is estimated to contain around 4 $\mu$M Mn$^{2+}$ and 230 $\mu$M Zn$^{2+}$ (335).

Genes encoding proteins involved in iron, Zn$^{2+}$ and Mn$^{2+}$ uptake have been subject of study in S. pneumoniae and these systems have also been implicated in virulence (48,49,79,80,211). At the same time, there are proteins encoded by the S. pneumoniae genome that play a role in export of metal ions, when the intracellular concentrations exceed certain thresholds, for example czcD, involved in the resistance against high concentrations of specifically Zn$^{2+}$ (chapter 5). Thus, Zn$^{2+}$ seems to have an important role in the physiology and habitat of S. pneumoniae and it is not surprising that there are transcriptional regulators that mediate the response to Zn$^{2+}$.

In bacteria, there are several families of metal-responsive transcriptional regulators that include Zn$^{2+}$ dependent regulators (120). The Fur family Zur repressors, which can be
classified in 3 subgroups, are well-studied Zn\textsuperscript{2+}-responsive transcriptional repressors involved in the regulation of high affinity Zn\textsuperscript{2+} uptake in many Gram-positive and Gram-negative bacteria (247). Another bacterial family of metalloregulators is DtxR, including Mn\textsuperscript{2+} and iron and Zn\textsuperscript{2+} responsive regulatory proteins. Third, certain streptococci contain a MarR family regulator, named AdcR, which regulates high affinity Zn\textsuperscript{2+} uptake as well as virulence genes (118,120,247). Transcriptional regulators that mediate Zn\textsuperscript{2+} resistance are often members of the MerR and SmtB/ArsR families, like CzrA in \textit{B. subtilis} and \textit{Staphylococcus aureus} (52,172,221,295).

In \textit{S. pneumoniae} a number of metalloregulators have been characterized to date. There are at least three transcriptional regulators encoded by the \textit{S. pneumoniae} genome that mediate the response to Zn\textsuperscript{2+}. The \textit{czcD} Zn\textsuperscript{2+}-resistance gene is activated in the presence of Zn\textsuperscript{2+} by the novel TetR family transcriptional regulator SczA, of which homologues can be found in several other related streptococci (chapter 5). Furthermore, the DtxR family regulator PsaR, of which homologues in other organisms have been reported to regulate gene expression in response to metal ions like Mn\textsuperscript{2+} and Fe\textsuperscript{3+} (23,267,300), regulates the expression of three virulence genes, encoding the Mn\textsuperscript{2+} ABC transporter PsaBCA, the extracellular serine protease PrtA and the choline binding protein PcpA in response to Zn\textsuperscript{2+} and Mn\textsuperscript{2+} (chapter 4). Zn\textsuperscript{2+} causes derepression of PsaR targets, whereas Mn\textsuperscript{2+} causes repression, which is likely the result of Mn\textsuperscript{2+}-dependent binding and Zn\textsuperscript{2+}-dependent release of PsaR from the target promoters. Exposing \textit{S. pneumoniae} to Zn\textsuperscript{2+} limitation results in the overexpression of several putative virulence genes, including genes encoding the pneumococcal histidine triad protein PhtA and the laminin binding protein Lmb, both located at the cell’s surface. These are likely to be regulated by the Zn\textsuperscript{2+}-dependent repressor AdcR (247), chapter 4). In \textit{Streptococcus suis}, AdcR has been shown to bind in a Mn\textsuperscript{2+}- and Zn\textsuperscript{2+}- dependent manner to the promoter regions of the \textit{adcRCBA} operon and a gene homologous to the pneumococcal \textit{lmb} gene (16). The same study also showed that immunization with Lmb provides a protective response to infection with \textit{S. suis}, and therefore it might be a good vaccine candidate. The large transcriptional response of \textit{S. pneumoniae} to Zn\textsuperscript{2+} limitation and Zn\textsuperscript{2+} stress, as well as the presence of several Zn\textsuperscript{2+} transport genes in the pneumococcal genome implies that Zn\textsuperscript{2+} is an important factor in the pneumococcus-host interaction.

Likely, also other trace metal ions contribute to a larger or smaller extent to pneumococcal physiology and virulence. A recent paper describes the response of \textit{S. pneumoniae} to iron restriction, showing that this had an effect on the expression of a variety of proteins, including glutamine synthetase, ferritin and PsaA (225). Strikingly, none of the iron uptake systems were significantly affected. The orphan response regulator RitR was shown to regulate one of the iron uptake systems, but apparently not depending on the iron concentration (328). Thus, iron-responsive regulators remain to be identified in the pneumococcal genome. Also Cu\textsuperscript{2+} metabolism has not yet been subject of study in \textit{S. pneumoniae}. However, \textit{S. pneumoniae} contains a \textit{cop} operon, harbouring the \textit{copY} Cu\textsuperscript{2+}-responsive regulatory gene, the Cu\textsuperscript{2+} uptake gene \textit{copA} and a putative cupredoxin gene \textit{cuA} (cupredoxins are involved in Cu\textsuperscript{2+} electron transfer) (263), suggesting that \textit{S. pneumoniae} also has to cope with changes in the extracellular Cu\textsuperscript{2+} concentration. It would therefore be interesting to investigate the response of \textit{S. pneumoniae} to physiological concentrations of Cu\textsuperscript{2+}. Thus, metal ions represent an important class of extracellular nutrients that modulate the physiology and virulence of \textit{S. pneumoniae}.

**Regulation of nitrogen metabolism**

One aspect of pneumococcal physiology that has remained poorly investigated until recently is nitrogen metabolism. Little is known about how \textit{S. pneumoniae} utilizes the nitrogen sources available in the different compartments of the human body it may inhabit
and how this relates to pneumococcal virulence. The most abundant proteins in serum are albumin and globulin, which are present in concentrations of about 40 g/L and 20 g/L, respectively. In addition, red blood cells contain hemoglobin, which in total blood is present in a concentration of around 150 g/L. Other minor nitrogen sources in the blood are free amino acids (around 200 mg/L, of which about one-third glutamine) and urea (max. 250 mg/L) (53).

A few studies have made some first steps toward unraveling the role and functioning of nitrogen metabolism in S. pneumoniae, and show that proteins involved in nitrogen metabolism contribute significantly to the virulence and fitness of S. pneumoniae. For example, the peptide uptake locus Ami-AliA/B is necessary for efficient nasopharyngeal colonization by pneumococcus but not for pneumococcal pneumonia (154). Moreover, recently the pleiotropic regulator of nitrogen metabolism CodY, which responds to the extracellular concentration of branched-chain amino acids and regulates genes encoding proteins involved in amino acid metabolism and virulence, including Ami-AliA/B and the choline binding protein PcpA (127), has been found to be essential for efficient nasopharyngeal colonization. Also in other Gram-positive pathogenic bacteria CodY has been found to regulate virulence gene expression and affects virulence features, like in S. aureus (205), Listeria monocytogenes (28,297), S. mutans (192) and S. pyogenes (206).

Recent studies have addressed the metabolism of glutamine and glutamate in pneumococcus. Glutamate and glutamine have a central position in nitrogen metabolism, as these amino acids are main nitrogen donors in the cell (Fig. 3). In S. pneumoniae, the glutamine synthetase gene glnA and the glutamate/glutamine transport genes glnPQ are regulated by the glutamine responsive regulatory protein GlnR, which represses transcription in the presence of a high concentration of glutamine (chapter 6). GlnR was originally discovered in B. subtilis, where it, together with a paralogous protein TnrA, which is not present in S. pneumoniae, governs glutamine metabolism (91). The activity of both GlnR and TnrA depends on GlnA (284-286,343). It was shown that the function of GlnR in S. pneumoniae depends on GlnA as well, which is caused by the a stimulatory effect of GlnA on the interaction with its target promoters (chapter 6), a phenomenon that was recently also observed in B. subtilis (93).
Expression of the GlnR target \textit{gdhA}, encoding glutamate dehydrogenase, is also regulated by CodY, suggesting that \textit{gdhA} is an important control point of nitrogen metabolism in \textit{S. pneumoniae} (127, chapter 6). Interaction between the CodY and GlnR regulons is also underscored by the observation that many CodY-regulated genes are strongly upregulated in a \textit{glnPA} double mutant (chapter 7). Also in the lactic acid bacterium \textit{Lactococcus lactis}, CodY and GlnR have a target in common, which is the ammonium utilization operon \textit{amtB-glnK} (180). However, the roles of \textit{gdhA} and \textit{amtB-glnK} in \textit{S. pneumoniae} and \textit{L. lactis}, respectively, are not fully understood.

Interestingly, several studies show that glutamine/glutamate metabolic genes, encoding glutamine synthetase, glutamine transport proteins and glutamate dehydrogenase are important for the virulence of pathogens like \textit{Salmonella typhymurium}, \textit{Neisseria meningitidis}, \textit{Mycobacterium tuberculosis} and \textit{Streptococcus pyogenes} (164,241,244,313,327). In \textit{S. pneumoniae}, STM screens have also identified \textit{glnQ} and \textit{glnA} to be important for virulence (181,254). A detailed investigation of the role of \textit{glnA} and \textit{glnP} for the virulence of \textit{S. pneumoniae} using several mouse infection models showed that these genes have host site-specific roles in virulence: \textit{glnA} contributes to survival during colonization of the nasopharynx, whereas \textit{glnP} is necessary for survival in the lungs. In the blood compartment, \textit{glnP} and \textit{glnA} are able to compensate for each others loss, but a \textit{glnPA} double mutant is completely avirulent (chapter 7).

Thus, the CodY and GlnR nitrogen metabolism regulons appear to be of significant importance for full virulence of pneumococcus. Future studies could address the metabolism of other important amino acids and its relation to virulence, such as arginine, which is possibly controlled by an unusual number of three ArgR/AhrC type regulators in \textit{S. pneumoniae} (178,179), and sulfur amino acids, which may be controlled by a CmbR orthologue as has been studied in \textit{L. lactis} (303). In addition, to be able to understand how this bacterium utilizes and processes the nitrogen sources present in the human body, it would be interesting to identify the substrates for the many amino acids and peptide transport systems, as well as peptidases and proteases encoded by the \textit{Streptococcus pneumoniae} genome (133).

In conclusion, we still only poorly understand the different ways in which \textit{S. pneumoniae} takes advantage of the nitrogen source in the human body and it would be worthwhile to extend the recent publications on this topic in order to get an integrated view of pneumococcal nitrogen metabolism and its importance for the virulence and fitness in different host-compartments.

### Outline of this thesis

The work described in this thesis contributes to the field of pneumococcal research in three respects. First, it describes the development of new and improved molecular and genetic techniques to study \textit{S. pneumoniae} (\textit{chapters 2} and \textit{3}). Second, it contains three chapters that deal with molecular characterization of transcriptional regulatory processes in \textit{S. pneumoniae} (\textit{chapters 4}, \textit{5} and \textit{6}). Third, the importance for pneumococcal virulence of one of the processes studied, namely glutamine metabolism, was investigated (\textit{chapter 7}).

\textbf{Chapter 2} presents the development of several tools that can be used to study the pneumococcus. These tools include a nisin-inducible overexpression system, a \textit{lacZ} based reporter system, a chemically defined medium and a system for the construction of unmarked mutations in the pneumococcal chromosome. These molecular tools were used throughout the work described in this thesis for the characterization of a number of pneumococcal genes.
In chapter 3 the development of a novel genome-wide screening technique called genomic array footprinting (GAF) is described that allows the identification of conditionally essential genes by means of a combination of random mutagenesis and microarraying. To be able to generate random mutant libraries in *S. pneumoniae*, both an *in vitro* and an *in vivo* method for transposon mutagenesis were optimized for use in *S. pneumoniae*. The transposable DNA cassettes were equipped with outward facing T7 RNA polymerase promoters, to be able to specifically amplify the chromosomal *S. pneumoniae* DNA adjacent to the transposon insertion sites. By subsequently using microarrays, loss or enrichment of transposon mutants in large mutant libraries could be detected. In a Zn$^{2+}$ stress experiment, the technique was shown to correctly identify *czcD* (see also chapter 5) as the main Zn$^{2+}$-resistance gene in *S. pneumoniae*.

In chapters 4 and 5 the genome-wide transcriptional response of *S. pneumoniae* to Zn$^{2+}$ was studied by using microarrays. Furthermore, the underlying regulatory mechanisms mediating this response were characterized. Chapter 4 demonstrates that the expression of three virulence genes, encoding the choline binding protein PcpA, the extracellular serine protease PrtA and the Mn$^{2+}$ uptake lipoprotein complex PsaBCA gets strongly upregulated in the presence of a high extracellular Zn$^{2+}$ concentration. Subsequently elevating the Mn$^{2+}$ concentration in the medium was found to again cause repression of these genes. It is shown that the DtxR family regulator PsaR is responsible for the counteracting effects of Mn$^{2+}$ and Zn$^{2+}$ on the expression of the virulence genes. Chapter 4 also contains an expression profile of *S. pneumoniae* under Zn$^{2+}$ limitation, showing that several virulence genes, encoding the laminin binding protein LmB, pneumococcal histidine binding protein PhtA and the Zn$^{2+}$-uptake system AdeBCA, are strongly upregulated in this condition. These genes are likely to be regulated by the transcriptional repressor AdeR.

In chapter 5, a novel and atypical transcriptional regulator, SczA, belonging to the TetR family was characterized. SczA is proven to function as a transcriptional activator of an operon including the Zn$^{2+}$-resistance gene *czcD*. An intriguing mechanism of transcriptional activation was revealed, in which SczA activates gene expression by Zn$^{2+}$ induced binding to an enhancer DNA sequence in the *czcD* promoter, whereas SczA binding to a second DNA motif ensures that the *czcD* promoter is fully shut down in the absence of Zn$^{2+}$.

The way in which *S. pneumoniae* responds to and utilizes another important environmental factor, the extracellular nitrogen source, is described in chapters 6 and 7. In chapter 6 the glutamine dependent transcriptional repressor GlnR is characterized on the molecular level. GlnR is shown to directly repress three operons, the *glnPQzwf* operon encoding a glutamine/glutamate ABC transport system as well as the glucose-6-phosphate dehydrogenase gene, the glutamate dehydrogenase gene *gdhA*, and the *glnRA* operon encoding GlnR and glutamine synthetase GlnA. Repression by GlnR is only effectuated in the presence of GlnA and sufficient glutamine or glutamate/NH$_4^+$. Chapter 7 describes the impact of GlnR and its regulon on the virulence of *S. pneumoniae*. Mutants in all components of the GlnR regulon were individually tested in mouse models of colonization, pneumonia and sepsis. The data indicate site-specific effects on virulence and fitness of *glnA* and *glnP* mutants and moreover that these genes are extremely important in maintaining nutritional homeostasis in the cell. Chapter 8 summarizes and discusses the most important findings.

In conclusion, this thesis contributes to the development of new technologies to study *S. pneumoniae* on the molecular level. Furthermore, it also provides novel insights in the molecular biology of *S. pneumoniae* and in the way in which it interacts with its environment and behaves in the host.