Swedish Institute for Infectious Disease Control and
Department of Microbiology, Tumor and Cell Biology,
Karolinska Institutet, Stockholm, Sweden

Genetic approaches towards understanding pneumococcal virulence and biology

Jenny Fernebro

Karolinska Institutet

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Swedish Institute for Infectious Disease Control

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ABSTRACT

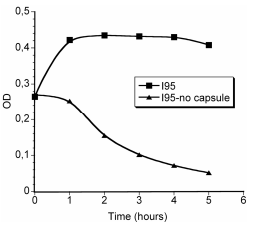
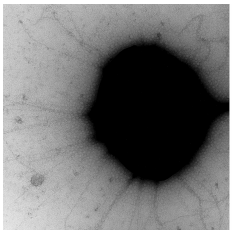
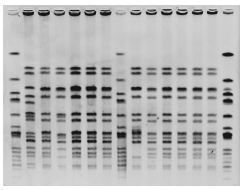
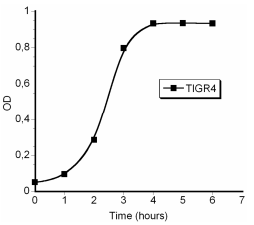
Streptococcus pneumoniae, the pneumococcus, is a major human pathogen giving rise to death and illness worldwide every year. It causes a wide variety of diseases, from normally harmless infections such as otitis media to potentially life-threatening systemic diseases such as pneumonia, meningitis and sepsis. However, it is commonly carried in the nasopharynx of healthy children. Pneumococcal infections are in general treated with antibiotics such as penicillin, but over the last decades antibiotic resistance has become an increasing problem. The aim of this thesis was to learn more about pneumococcal virulence as well as the mechanisms leading to resistance.

First, we studied the lytic behavior of pneumococcal clinical isolates, and found a link between the degree of lysis and the capsular serotype. Lytic antibiotics such as penicillin and vancomycin kill pneumococci partly by activating the pneumococcal autolysin LytA. We showed that strains of serotypes 1, 4, 6B and 23F were generally less lytic towards penicillin than other isolates. In addition, the isolates belonging to serotype 9V were the only ones showing reduced lysis towards vancomycin. Nonencapsulated strains were also more lytic than encapsulated ones, both upon addition of lytic antibiotics and in the stationary phase.

In the second study we identified a novel pneumococcal virulence factor, the pilus. This molecule is encoded by the *rlrA* pathogenicity islet, containing three structural genes (*rrgA-C*), three sortases (*srtB-D*) and a positive regulator (*rlrA*). With electron microscopy and immunogold staining we could show that pili are mainly built up by RrgB, with RrgA located at the base and RrgC present on the tip of the structure. Mutants lacking pili were less virulent and caused less inflammation in an animal model. The importance of pili was further established in the third study, where we looked at the distribution of pneumococci nonsusceptible to penicillin (PNSP) in Sweden. Between 1999 and 2003 the frequency of serotype 14 increased from 12% to 26% of all PNSP. By investigating a large number of PNSP isolates of types 9V, 14 and 19F with multilocus sequence typing and pulsed-field gel electrophoresis we concluded that a serotype switch had occurred with a 9V clone obtaining the type 14 capsule. The rise seen in PNSP of serotype 14 could be attributed to this clone, known as Spain^{9V}-3 of ST156. We also showed that about 50% of all PNSP in 2003 belonged to this clonal cluster and that its success may be explained by the fact that it carries the *rlrA* islet and expresses pili. Two type 19F isolates of ST156 were studied in a mouse model of colonization. These isolates were identical as shown by microarray, except that one of them was piliated. The piliated strain outcompeted the nonpiliated one, again showing the advantage of piliation in colonization.

In the final study we correlated *in vitro* fitness to *in vivo* virulence. The fitness of a panel of clinical isolates with known virulence in mice was monitored. Two isolates each of types 1, 6B, 7F, 14 and 19F were included, chosen to be as genetically different as possible. Only one of the isolates, a type 14 isolate, had a growth rate much slower than the rest. Interestingly, the type 14 strains differed the most in virulence, when comparing the strains within each serotype pair to each other. The slow-growing strain was less virulent than the other type 14 isolate in a mouse model of infection. To further investigate the effect of fitness defects on virulence, five mutants were constructed and analyzed. These were mutated in five well-conserved house-keeping genes, resulting in growth defects for three of them. These three mutants were, to different degrees, attenuated in virulence in an intranasal mouse model. We conclude that pneumococcal fitness is of great importance for *in vivo* virulence.

THESIS AT A GLANCE

	Question	Method	Result and conclusion	
I	How does the pneumococcal capsule influence the spontaneous and antibiotic-induced lytic behavior?	The antibiotic-induced lytic response of clinical isolates of serotypes 1, 3, 4, 6B, 9V, 14 and 23F was monitored. Also, nonencapsulated mutants in three serotypic backgrounds were evaluated.	Specific capsules affect lysis towards penicillin and vancomycin differently. Serotype 1, 4, 6B and 23F isolates were shown to be less lytic towards penicillin than the other isolates. Also, isolates belonging to serotype 9V were the only ones with reduced lysis to vancomycin. Nonencapsulated strains were generally more lytic than encapsulated ones.	
II	What is the function of the pneumococcal *rlrA* pathogenicity islet?	The *rlrA* islet was deleted in two isolates of types 4 and 19F and inserted into a nonpiliated type 2 strain. Mutants and wild-types were evaluated using methods such as EM, Western blotting and animal models.	The *rlrA* islet was found to encode a pneumococcal pilus. This was shown both with EM and Western blotting. The pilus polymers are mainly built up by RrgB subunits. Mutants deficient in the *rlrA* islet do not produce pili and adhere less to lung epithelial cells. They also cause less invasive disease in mice and evoke lower levels of the proinflammatory cytokines IL-6 and IFN.	
III	What is the molecular epidemiology behind the spread and success of penicillin nonsuseptible pneumococcal (PNSP) clones?	PNSP of serotypes 9V, 14 and 19F were investigated using pulsed-field gel electrophoresis and multilocus sequence typing. Some selected isolates were further analyzed using microarray and animal models.	The rise in PNSP of serotype 14 could be explained by clonal expansion of a capsular switch variant of ST156, previously found manly among the dominating type 9V in Sweden. More than 50% of Swedish PNSP in 2003 were shown to belong to this clonal cluster. We suggest that expression of pili is one important factor for its successful spread.	
IV	What is the impact of *in vitro* fitness defects on the ability of pneumococcal clinical isolates and mutants to colonize and cause invasive disease in mice?	Clinical isolates with known disease potential in mice were analyzed for *in vitro* fitness. Deletion mutants in five house-keeping genes were constructed and evaluated for *in vitro* growth as well as in an intranasal animal model.	Small changes in *in vitro* fitness cause attenuation in virulence *in vivo*. A clinical isolate of type 14 was shown to grow much slower than the other isolates and was also less virulent than the other type 14 isolate studied. An association between *in vitro* growth and *in vivo* virulence was found while studying the mutants. However, most of the slow-growing strains were still able to colonize mice.	

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. **Fernebro, J**, Andersson, I, Sublett, J, Morfeldt, E, Novak, R, Tuomanen, E, Normark, S, Henriques Normark, B.
Capsular expression in *Streptococcus pneumoniae* negatively affects spontaneous and antibiotic-induced lysis and contributes to antibiotic tolerance.
J Infect Dis, 2004, 189(2), 328-338.

II. Barocchi, MA*, Ries, J*, Zogaj, X*, Hemsley, C, Albiger, B, Kanth, A, Dahlberg, S, **Fernebro, J**, Moschioni, M, Masignani, V, Hultenby, K, Taddei, AR, Beiter, K, Wartha, F, von Euler, A, Covacci, A, Holden, DW, Normark, S, Rappuoli, R, Henriques Normark, B.
A pneumococcal pilus influences virulence and host inflammatory responses.
Proc Natl Acad Sci USA, 2006 Feb 21;103(8):2857-62.

III. Sjöström, K*, Blomberg, C*, **Fernebro, J**, Dagerhamn, J, Morfeldt, E, Barocchi, MA, Andersson, M, Browall, S, Moschioni, M, Albiger, B, Henriques, F, Rappuoli, R, Normark, S, Henriques Normark, B.
Clonal success of piliated penicillin nonsusceptible pneumococci.
Proc Natl Acad Sci USA, 2007 Jul 31;104(31):12907-12.

IV. **Fernebro, J**, Blomberg, C, Morfeldt, E, Wolf-Watz, H, Normark, S, Henriques Normark, B.
The influence of *in vitro* fitness defects on pneumococcal ability to colonize and to cause invasive disease.
Submitted manuscript.

*The authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

bp	Base pairs
CBP	Choline-binding protein
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
EM	Electron microscopy
kb	Kilo bases
kDa	Kilo Daltons
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
OD	Optical density
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PG	Peptidoglycan
PNSP	Penicillin nonsusceptible pneumococci
TA	Teichoic acid
TNFα	Tumor necrosis factor α
WHO	World Health Organization

1 INTRODUCTION

1.1 GENERAL ASPECTS OF STREPTOCOCCUS PNEUMONIAE

Streptococcus pneumoniae, or the pneumococcus, is a gram-positive bacterium, seen mostly as diplococci or in short chains. It is alpha-hemolytic and can be identified due to its sensitivity to optochin and bile (bile solubility test) [1]. It is naturally transformable, i.e. it is able to take up DNA from its surroundings and incorporate it into its genome. This is regulated by a quorum-sensing mechanism, involving the production and sensing of the competence-stimulating polypeptide (CSP) [2].

Streptococcus pneumoniae was first discovered in 1880, by Pasteur and Sternberg independently [3, 4]. In both cases, human saliva was inoculated into rabbits and pneumococci were isolated from the animals. Soon after, the bacterium was associated with pulmonary disease as well as extrapulmonary disease, with pneumococci isolated from lungs, blood and other body fluids. One of the first methods used to identify pneumococci was the Quelling test [5]. Mixing a pneumococcal sample with capsule-specific antibodies makes the bacterial colonies appear to swell, while watched through a phase-contrast microscope. In the beginning of the 20th century it was already known that the pneumococcal capsule was an important virulence factor and that antibodies towards it were protective. Soon, it was also documented that pneumococci could be divided into different serotypes based on the capsular structures [6, 7]. In 1944, Avery showed that a nonencapsulated, avirulent pneumococcal strain became encapsulated and virulent after the addition of nucleic acid from another strain [8]. This was the first time that the role of DNA as the carrier of inheritable traits was recognized. Since then, much more has been learnt about *Streptococcus pneumoniae* and the pneumococcal genome. In 2001, the complete genomes of a virulent serotype 4 strain (TIGR4) and the avirulent, nonencapsulated laboratory R6 strain were published [9, 10].

Pneumococcal infections have been treated mainly with antibiotics since the introduction of sulfonamides in the 1930s. For a long time penicillin has been the most commonly used drug and in Sweden it still is. However, as early as 1943 pneumococcal resistance to penicillin was reported after treatment of mice [11], and in 1967 it was also reported after treatment of man [12]. Although most pneumococcal infections still are treatable with penicillin, drug resistance is a growing problem that probably will have a large impact on the future treatment of pneumococcal disease. Therefore, it is crucial that different treatment regimes are developed and that more effort is put into the prevention of pneumococcal infections. A heptavalent pneumococcal conjugated vaccine (PVC7) is widely used today and several more vaccines are under development. Good results have initially been reported for PVC7, but it has its limitations (discussed in the vaccine section).

Although research into this pathogen has lead to a greater understanding in most areas concerning pneumococcal disease, it remains a main cause of death and morbidity. The World Health Organization (WHO) estimates that 1.6 million people died due to pneumococcal disease in 2002 and the morbidity number is many times higher [13].

1.2 VIRULENCE FACTORS

A wide variety of pneumococcal virulence factors have been identified over the years. Major ones are the polysaccharide capsule, cell wall fragments, pneumolysin and choline-binding proteins. A number of large-scale studies have been performed to identify novel virulence factors, using methods such as signature-tagged mutagenesis, differential fluorescence induction analysis and microarray analysis [14-18]. These studies have generated an unexpectedly large number of pneumococcal genes, proposed to be essential for *in vivo* virulence and *in vivo* growth. It would be surprising if all these genes really encoded "true" virulence factors. Only the most well-known virulence factors will be discussed in this section.

1.2.1 Polysaccharide capsule

The major virulence factor of pneumococci is the polysaccharide capsule. It is present in basically all clinical isolates and mutants lacking capsule are regarded as avirulent [6]. Ninety different capsular types (serotypes) have been described [19], based on the chemical structure of the capsules. They contain a mix of repeating units of sugars, and vary in complexity. The pneumococcal capsule inhibits complement-mediated opsonisation, probably by blocking access to complement factor C3c deposited on the cell wall. Studies have also shown that the capsule is not only necessary in disease, but for colonization as well [20, 21]. However, during invasion of host cells the expression of capsule seems to be down-regulated [22]. Antibodies elicited towards the capsule are protective. However, since capsular antigens evoke a T-cell-independent immune response, infants cannot produce capsule-specific antibodies.

The capsular biosynthesis locus has a similar organization in almost all pneumococcal serotypes studied so far (figure 1). It is flanked by the same two genes in all strains; *dexB* upstream of the locus and *aliA* downstream of the locus. However, these genes are not involved in capsule formation. The first two genes in the locus, *cpsA* and *cpsB*, are the same in most serotypes and the following two genes are well conserved amongst different serotypes. The exceptions to this organization are type 3 and type 37. Type 3 contains variants of the standard four genes in the beginning of the operon, but these are not involved in the production of the type 3 capsule. Instead, the following two genes in the locus are responsible for capsule assembly [23]. Type 37 has an even more peculiar capsular arrangement. These strains contain the full capsular operon of type 33F, but due to mutations it is not active and capsule is produced from a single gene, *tts*, located elsewhere on the chromosome [24]. Since the biosynthesis locus is so well conserved, capsular switches are known to take place [25-28]. Genetic exchange can occur very close to the *aliA* and *dexB* genes, but may also

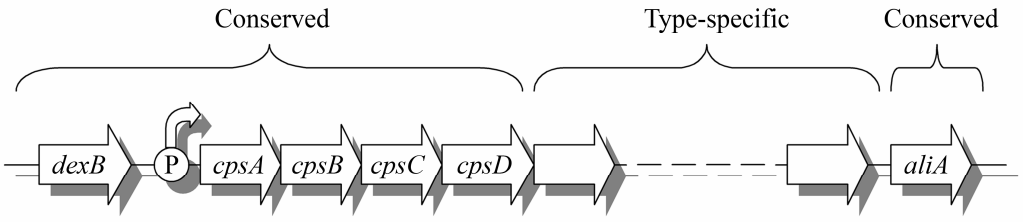


Figure 1. The arrangement of the capsular biosynthesis locus

include larger fragments of DNA [26, 29].

The regulation of capsular production is not fully understood. A type of on-off switching has been observed for isolates of types 3, 8 and 37 [30, 31]. For type 3 isolates this was seen while the bacteria were grown in a biofilm-like fashion, mimicking nasopharyngeal carriage. Nonencapsulated mutants arose spontaneously in the culture and loss of capsule could be explained by duplications in one of the capsular genes. Similar duplications were detected in types 8 and 37 isolates too and the phenomenon was shown to be reversible. The clinical relevance of these observations remains to be demonstrated. Capsular production can also be controlled via phase variation between the opaque and transparent stages. Colonies of the opaque form express more capsule and are more frequently isolated from blood [32].

In recent years it has been debated whether the capsular type or the clonal type is the major virulence determinant of pneumococcal isolates. Evidence for both sides has been presented [33], but most studies conclude that it is a mixture of both properties that determine virulence [34-36]. Kelly *et al*. studied the effect of capsular switches on virulence in mice [37]. Different genetic backgrounds were transformed with the type 3 capsule and virulence of the mutants was compared with that of the parental strains. For some of the strains the virulence was unchanged, for others it was enhanced and for some it was reduced, showing that it is the combination of capsule and genetic background that determines the virulence. However, some serotypes are frequently more associated with high mortality, such as type 3, while others cause less serious disease, such as type 1 [38, 39].

1.2.2 Cell wall fragments

Streptococcus pneumoniae has a typical gram-positive cell wall (figure 2). It is built up by several layers of peptidoglycan (PG), forming a rigid three-dimensional network. The PG contains many molecules unique to this structure, such as D-alanine and D-glutamic acid, but the main components are *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG). These two amino sugars, alternated and connected by β1-4-linkages, make up the glycan backbone of PG. The glycan chains are then further cross-linked

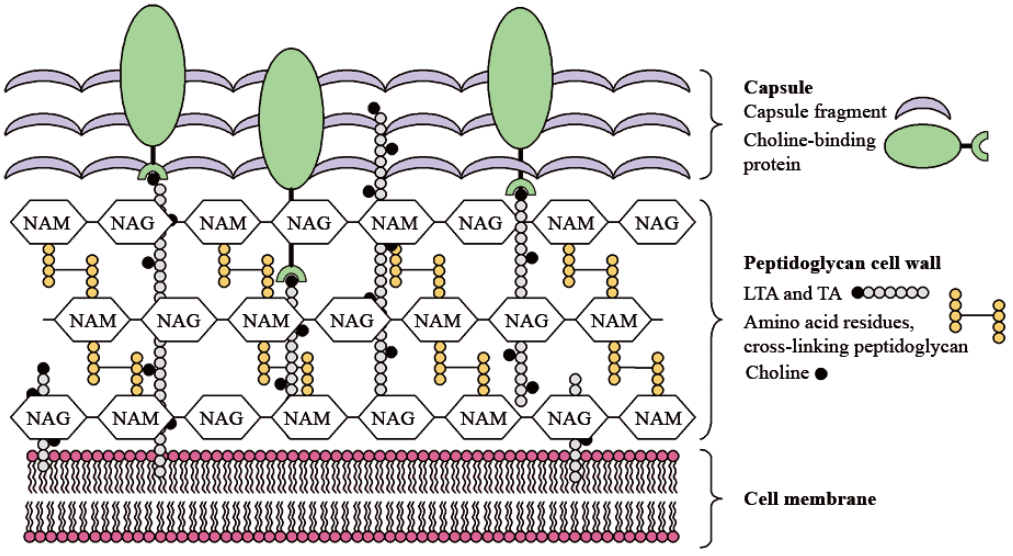


Figure 2. Structure of the pneumococcal cell wall.

via stem peptides between the NAM residues. The pneumococcal cell wall is rich in teichoic acid (TA, also called C-polysaccharide) and lipoteichoic acid (LTA) [40]. These structures are principally the same, but LTA is hydrophobically anchored to the lipids of the cell membrane, while TA is covalently attached to the peptidoglycan [41]. Another important feature of the pneumococcal cell wall is choline. This molecule is covalently linked to TA and LTA and is a requirement for pneumococcal growth [42, 43]. Many pneumococcal proteins are anchored to choline residues, hence the name choline-binding proteins (CBPs). Most of these proteins are of unknown function, but some have been shown to be involved in virulence and will be discussed in the next section.

It has been shown that pneumococcal cell wall preparations trigger inflammation [44, 45]. Purified cell wall stimulates human monocytes to produce TNFα, but it takes about 1000 times more of this substance than of lipopolysaccharide (LPS) to elicit the same response [46, 47]. Majcherczyk *et al*. digested pneumococci with the major pneumococcal autolysin LytA, fractionated the lysate and investigated these fractions [47]. The stimulatory capacity of the total lysate was not greater than for the whole cell wall preparation, but some of the fractions elicited stronger responses. After analysis of the fractions the authors concluded that small monomeric or dimeric stem peptide structures were not proinflammatory, while larger pieces such as trimeric peptides were highly active, with stimulatory properties comparable to LPS.

1.2.3 Surface proteins

This section deals with some of the pneumococcal protein virulence factors. Pneumolysin and the autolysins are discussed in separate sections.

It is estimated that pneumococci possess more than 500 surface proteins [48], many of which are choline-binding proteins. The CBPs all contain several repeats (4-11) of a choline-binding domain made up by 20-22 amino acids [49]. It is situated at the carboxyl end of the proteins, except for in LytB and LytC, where it is expressed at the amino terminus [50].

Choline-binding protein A, **CbpA**, also known as SpsA (*Streptococcus pneumoniae* secretory IgA-binding protein) and PspC (pneumococcal surface protein C) is the most abundant choline-binding protein [51]. It has been suggested to be involved in immune evasion, adherence and invasion. Mutants lacking CbpA are unable to establish colonization in an infant rat model and are defective in binding to cytokine-activated human cells [51]. CbpA has been shown to adhere to several human molecules, such as the glycoconjugates sialic acid and lactotetraose [51] and the complement proteins C3 and factor H [52, 53]. It also binds the secretory component (SC) of secretory immunoglobulin A (sIgA) and the polymeric immunoglobulin receptor (pIgR) [54]. Normally, pIgR transports antibodies across epithelial cells. It has been proposed that by binding to pIgR the bacterium can promote its transportation over the mucosal barrier, but contradicting data have been reported [55, 56].

PspA (pneumococcal surface protein A) is a choline-binding protein with homology to CbpA. Mutants lacking PspA are attenuated in animal models and the major proposed function for the protein is to inhibit complement activation [57, 58]. PspA interferes with complement factor B, thereby preventing the deposition and processing of C3b [59]. It has also been suggested that PspA is involved in iron acquisition, since it binds human lactoferrin [60]. As antibodies towards PspA are protective, the protein is a potential vaccine candidate [57].

Neuraminidase A, **NanA,** has been shown to affect colonization in a chinchilla model, possibly by cleaving off *N*-acetylneuraminic acid (sialic acid) from mucin, glycoproteins and oligosaccharides [61]. This would decrease the

viscosity of the mucus and make potential binding sites on the cell surface more accessible. Two other proteins also implicated in pneumococcal colonization are **PsaA** (pneumococcal surface antigen A) and **PavA** (pneumococcal adherence and virulence factor A). PsaA is a lipoprotein that is important for adherence and virulence [62], indicated by the fact that specific antibodies are protective [63]. PavA binds fibronectin and has been proposed to be involved in both adherence and invasion [64, 65].

1.2.4 Pneumolysin

Pneumolysin is a cytotoxin that shares amino acid homology with hemolysins in other gram-positive bacteria, such as *Streptococcus pyogenes* and *Listeria monocytogenes*. It is a 53-kDa cytoplasmic protein that is present in almost all clinical isolates [66] and deleting it leads to reduced virulence in mice [67]. Its effect on different tissues and cell lines has been evaluated in several studies. When injected straight into the lungs of rats, it induces pathology similar to that seen in pneumococcal infections [68]. If added to pulmonary alveolar epithelial cells, it is highly cytotoxic and increases the permeability of the cells [69]. Previously, pneumolysin was thought to be released only during lysis of the bacteria, but in 2001 it was suggested to be secreted by some other mechanism as well [70]. Pneumolysin is believed to have multiple functions in virulence, as the toxin has both cytotoxic and proinflammatory properties. It binds host cell cholesterol and forms pores in the membrane, disrupting the cells. Also, pneumolysin can bind to the Fc portion of nonspecific antibodies, thereby activating the classical complement pathway, leading to inflammation and tissue damage [71]. Although many studies have established pneumolysin as an important virulence factor, the picture is not completely clear. Recently, clinical isolates have been detected that express pneumolysin without hemolytic effect [72] or do not express pneumolysin at all [73]. Also, a pneumolysin mutant in TIGR4 produced in our laboratory was not attenuated after intranasal challenge of mice (unpublished data).

1.2.5 Autolysins

Streptococcus pneumoniae is known to undergo spontaneous autolysis in the stationary phase (T4R in figure 3). This is attributed to the major autolysin, an amidase known as LytA. During growth this choline-binding protein is expressed, but is somehow inhibited, allowing the cells to reach stationary phase. It then becomes active and lyses the bacteria by targeting covalent bonds of the cell wall. Specifically, LytA cleaves the amide bond between *N*-acetylmuramic acid and the first amino acid of the stem peptide, L-alanine. A LytA-deficient mutant does not lyse in stationary phase (T4RΔLytA in figure 3) [74].

Antibiotic-induced lysis is also dependant on LytA activity. Penicillin and other lytic antibiotics trigger a lytic response in pneumococci by somehow inducing LytA. This is, however, not the only killing mechanism, as a LytA-deficient mutant is also killed by penicillin [75].

Several biological functions have been

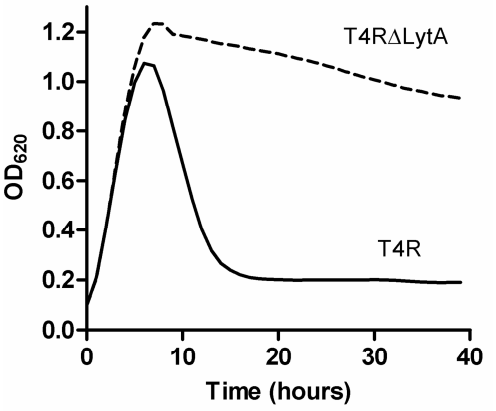


Figure 3. Spontaneous lysis of T4R and the LytA knockout of this strain.

reported for LytA. Mutants lacking LytA are less virulent in mouse models [76, 77]. It has been proposed that autolysis is responsible for some of the inflammatory response that is elicited during pneumococcal inflections. It is likely that the cell wall fragments shed in the process make a contribution to the inflammation. Autolysis may also be of importance to generate material for genetic exchanges. Pneumococci are naturally transformable and can evolve by obtaining DNA shed by other strains after autolysis. Finally, LytA is the cause of bile solubility; one of the best ways to diagnose pneumococci. Deoxycholic acid, one of the bile components, activates the autolysin so that the bacteria lyse upon bile addition. Some bile-resistant strains do exist, containing variants of the autolysin [78].

Pneumococci express two more cell wall hydrolases besides LytA; LytB and LytC. These are also choline-binding proteins. LytB is responsible for cell separation during pneumococcal growth and its inactivation leads to the formation of long chains [79]. LytC seems to work as an autolysin in the stationary phase at 30°C [50]. This may have implications on colonization, since the nasopharyngeal temperature is usually lower than the normal body temperature of 37°C.

1.2.6 Phase variation

In 1994, Weiser *et al*. showed that *Streptococcus pneumoniae* undergoes reversible phase variation between an opaque form and a transparent form [80]. Since then, the impact of phase variation on pneumococcal adherence and virulence has been studied in detail. Colonies of the opaque form are larger, whiter and more uniform than their transparent counterparts [80]. They undergo less spontaneous lysis, express more polysaccharide capsule and express less teichoic acid [32, 81]. Also, they are more virulent than the transparent variants in systemic infections in animal models and are more frequently isolated from human invasive disease [32, 82]. Transparent colonies, in contrast, adhere better to numerous human cell types *in vitro* and are more potent colonizers in animal models [80, 83]. Specifically, they bind GlcNAc much stronger than the opaque variants; GlcNAc being a main receptor during nasopharyngeal colonization [83]. Also, they express more pyruvate oxidase (SpxB), a protein shown to be of importance for H_2O_2 production and colonization [84]. The genetic background of phase variation is not fully understood. One study identified repetitive intergenic elements that were necessary for expression of phase variation at high frequency [85].

1.3 GRAM-POSITIVE PILI

Pili are long, hair-like structures expressed on the bacterial surface. The first gram-positive pili reported were those of *Corynebacterium* species [86]. These structures differ from the gram-negative variants that had been described previously by the fact that the subunits are covalently linked to each other as well as to the cell wall. Also, specific membrane-bound transpeptidases, sortases, are needed for their assembly. By now, pilus expression has been described in several *Actinomyces* spp. as well as *Streptococcus* spp. [87-91]. Typically, the pilus structure is build up by multiple copies of one subunit, with one or two accessory proteins decorating the tip, the base or the shaft of the molecule. Often, the subunit at the tip of the pilus acts as an adhesin. The subunits are secreted through the cell membrane by the Sec machinery and polymerization is catalyzed by the sortases. All known subunits have the LPXTG motif (or a similar motif), recognized by the sortases [92].

Several types of gram-negative pili have been described. For example, some *E. coli* strains express type I pili that mainly function as adhesins. Type IV pili, expressed by *E. coli*, *Pseudomonas* and *Neisseria* species amongst others, may act as adhesins but can also transfer genetic material. For gram-positives, the pili investigated this far seem to function primarily as adhesins [88, 93, 94]. In several cases it has been shown that immunization with pilus subunits mediate protection in animal models, making these molecules attractive vaccine candidates [95, 96].

In 2003, Ton-That and Schneewind published the organization of pili in *Corynebacterium diphtheriae* [97]. Due to sequence homology they proposed that similar structures may be produced by pneumococci as well. Specifically one pneumococcal protein was indicated to be involved in pilus assembly; the RrgB protein. The gene encoding RrgB is part of the pneumococcal ***rlrA* pathogenicity islet**. The importance of the *rlrA* islet in pneumococcal virulence was first reported in 2002 [14]. The islet was later shown to be controlled by a positive regulator, RlrA, and a negative regulator, MgrA, located elsewhere on the chromosome. They control the expression of six genes within the islet; three sortases (SrtB, SrtC and SrtD) and three proteins (RrgA, RrgB and RrgC) with homology to the LPXTG family of cell wall-anchored surface proteins [98, 99]. These are transcribed from several promoters, one for the sortases, one for RrgA and one for RrgB and RrgC (figure 4) [98]. Even though studies have shown the importance of the *rlrA* pathogenicity islet, the biological function of it remains unknown.

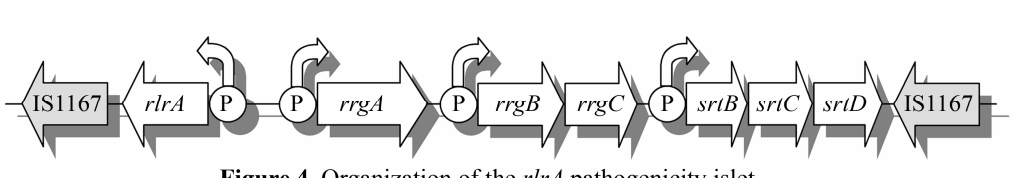


Figure 4. Organization of the *rlrA* pathogenicity islet.

1.4 PNEUMOCOCCAL CARRIAGE AND DISEASE

The pneumococcus is one of the most important human-specific pathogens. Every year it kills more than 1.6 million people; most of them children in developing countries [13]. It causes a wide variety of disease, stretching from uncomplicated otitis media to life-threatening meningitis. However, the challenge in understanding pneumococcal biology lies in the fact that most children in day-care harbor the bacterium in their nasopharynx at some time without showing any symptoms at all. What causes the transition from harmless carriage state into invasive disease?

1.4.1 Carriage

Since pneumococci are strictly human-specific the asymptomatic carriers represent the only reservoir for the bacterium. Studies have shown that up to 40% of healthy children in day-care centers are colonized with *Streptococcus pneumoniae* [100]. Also, parents of those children are colonized to a higher extent than the rest of the population; 18-29% compared to 6% [101]. Colonization initially occurs at about 6 months of age and during the following years several pneumococcal strains will come and go and even colonize the child at the same time. Carriage can be intermittent or last for weeks or months [102].

1.4.2 Pathogenesis

Basically all pneumococcal infections start with colonization of the nasopharynx. Factors that have been shown to promote binding to epithelial cells include CbpA, NanA and choline [51, 61, 103]. The target molecules on the epithelial cells are usually sugars such as disaccharides and sialic acid, but also the platelet-activating factor (PAF) receptor can be used [103]. This receptor recognizes choline residues on the PAF molecule, which are also present on pneumococci. Once colonization has been established, it has been proposed that the bacteria can invade the epithelial cells by means of the polymeric Ig receptor (pIgR) [56]. The proposed mechanism is that CbpA binds to the receptor and that the bacterium is translocated across the mucosal epithelial cell layer, but this theory has been debated [55]. Once the bacteria reach the blood-stream numerous factors are needed. The most essential is the antiphagocytic polysaccharide capsule, but PspA, CbpA and pneumolysin are also of importance by interfering with complement activation [52, 59, 71]. Pneumococcal pneumonia is often associated with deficiency in bacterial clearance. Influenza virus, for example, may kill the ciliated epithelial cells of the upper respiratory tract, making it possible for pneumococci to gain access to the lungs. A viral infection may also cause epithelial cells to upregulate the PAF receptor, giving the bacteria further advantage to adhere to the cells. The tissue destruction often seen in pneumococcal pneumonia is mainly due to inflammation. When pneumococci gain access to the lungs, alveolar macrophages gather in an attempt to combat the bacteria. The polysaccharide capsule prevents phagocytosis and the bacteria can continue to multiply. Some may lyse, spreading inflammatory cell wall fragments and attracting more immune cells to the site.

1.4.3 Disease

Otitis media is the most common clinical manifestation of pneumococci. At the age of two years most children have experienced at least one pneumococcal otitis [104]. The symptoms are pain in the ear and fever. Complications that may occur include chronic otitis media, meningitis and hearing defects.

The clinical manifestation of **pneumonia** is a rapid onset of symptoms like fever, shaking chills, a productive cough, blood in sputum and shortness of breath. Due to tissue destruction,

the alveolar gas exchange is damaged, resulting in lack of oxygen and cyanosis. In 30% of the cases of pneumococcal pneumonia the bacteria spread to the blood causing sepsis [105].

Sepsis is defined as bacteremia, bacteria in the blood, with clinical manifestations of systemic inflammation. If it is not treated, sepsis can lead to multisystem organ failure and death.

Since the introduction of the Hib (*Haemophilus influenzae*, type B) vaccine in 1990, pneumococcus has taken over as the main causative agent of **meningitis** [106]. Although the same kind of disease can be caused by *Neisseria meningitidis* and Group B streptococci, the pneumococcal infections tend to be more severe and have the highest case fatality rates [106]. Meningitis is defined as the inflammation of the meninges, the membranes covering the brain and spinal cord. It is not fully understood how pneumococci cross the blood-brain barrier to cause meningitis. The clinical symptoms of bacterial meningitis are headache, fever, vomiting and neck stiffness. Serious complications of the disease that may follow are hearing loss, blindness, paralysis and death.

1.4.4 Immune response

Most symptoms of pneumococcal disease are generated by a host inflammatory response. Therefore, the interplay between the bacteria and the host immune response is of particular importance for the outcome of this infection. When a pathogen enters the human host the first line of defence is the **innate immune response**. This is based on the recognition of molecules that are highly conserved amongst pathogens, for example bacterial peptidoglycan. It involves cellular mediators such as macrophages and neutrophils, as well as soluble factors such as cytokines and the complement cascade. Specific recognition is accomplished via receptors; the main ones being the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD) receptors.

Several TLRs have been implicated to mediate recognition of pneumococcal components *in vitro* and *in vivo*. TLR4 has been shown to recognize pneumolysin [107]. TLR2 recognizes pneumococcal LTA as well as peptidoglycan and plays a role in models of meningitis [108, 109]. Recently, our group showed that TLR9-deficient mice were more prone to develop disease in an intranasal infection model [110]. However, mice deficient in TLR1, TLR2, TLR4 or TLR6 were not more susceptible to pneumococcal infection, suggesting that they play a redundant role.

During a pneumococcal infection, the complement system plays a major role in clearing the bacteria. The activation and binding of complement components to the bacterial surface leads to opsono-phagocytosis. For pneumococci, the classical pathway of activation is the most important one, with complement being activated by antibody-antigen complexes or the binding of acute-phase proteins, such as C-reactive protein, to the bacterium [111].

The innate immune system is also the link to the **adaptive immune response**. Specific lineages of T-cells and B-cells are activated upon antigen presentation by macrophages and other immune cells, leading to the clonal expansion of cells capable of mediating a specific antibody or cellular immune response to the invader.

1.5 PREVENTION AND TREATMENT

1.5.1 Drugs

The most commonly used drug in treating pneumococcal pneumonia is penicillin. Since the emergence of penicillin-resistant strains, erythromycin, tetracycline and other antibiotics have been more commonly used, but also these have led to development of resistance. However, no resistance has been reported, so far, to vancomycin.

When it comes to treatment of meningitis the picture is a bit more complicated. Since antibiotics lyse the bacteria, the drugs can lead to an inflammatory response, devastating to the patient. Therefore, it has been suggested that anti-inflammatory drugs should be administered at the same time as the antibiotics [112]. Another problem with meningitis is that the drugs do not pass the blood-brain barrier. If inflammation has damaged the area, drugs may leak through, but this may not be enough.

1.5.2 Vaccines

The first pneumococcal vaccine that was licensed was introduced in 1978. It was followed by a 23-valent variant in 1983, containing polysaccharides of the 23 most important serotypes (PPV23). This vaccine has a low efficacy in adults and performs even worse in children and the elderly. Infants cannot mobilize a robust response towards T cell-independent antigens, including poly-saccharides. Therefore, conjugated vaccines have been developed with proteins coupled to the polysaccharides, giving a better immune response. The vaccine that is primarily used today is the seven-valent pneumococcal conjugated vaccine, PCV7 (Prevenar^{TM}). It contains polysaccharide structures of types 4, 6B, 9V, 14, 18C, 19F and 23F, linked to the nontoxic diphtheria variant protein carrier CRM_{197}. It was licensed in 2000 and is currently included in the vaccine program in 14 countries [113], but is not universally recommended by WHO. In developing countries, serotypes 1 and 5 are responsible for a large proportion of severe disease and these are not included in the vaccine [114]. However, WHO has recommended that if the serotypes of the vaccine match the distribution in a certain country, the vaccine should be considered for inclusion in the childhood vaccination programs.

Studies performed in countries where PCV7 has been included in the vaccination program have shown good results. As the introduction of the vaccine has occurred quite recently in most countries, not all effects have yet been fully evaluated. Most of the published evaluations are based on data from the United States, where the vaccine was introduced in 2000. There, the incidence of invasive pneumococcal disease declined by 75% between 1998/1999 and 2003 within the vaccinated group of children <5 years of age [115]. When looking at only the serotypes included in the vaccine, the decrease was 95%. In the rest of the population, i.e. people older than 5 years, fewer cases of disease than expected were observed, showing a pronounced herd effect. During these years it was estimated that about 30,000 cases of vaccine-serotype invasive disease were prevented, while disease caused by nonvaccine-serotypes increased by about 5,000 cases, giving a net reduction of 25,000 cases. These data are supported by a recent study, showing that hospital admissions in the United States due to pneumonia in the vaccinated age group had declined by 39% between 1997 and 2004 [105]. Similar results as that seen for the United States can be seen in other countries, for example Australia [116] and Canada [117].

Since healthy carriers are the reservoir of pneumococcal strains, it is also important to monitor the effect of vaccination on carriage. Several studies have shown that although the rates of vaccine-type strains are dramatically reduced, the overall number of pneumococcal

isolates remains the same [118, 119]. The replacement can partly be explained by expansion of previously known clones of nonvaccine-types, but also clones that have not been seen before have emerged [120].

For several reasons, a new vaccine target is needed that is noncapsular. Firstly, it would protect against all strains and not only against a limited number of serotypes. The serotype distribution varies a lot, both geographically and in different demographic groups. Therefore, large differences in coverage rate of the vaccine have been reported, ranging from 50 to 85% in different age groups in European countries [121]. Also, although a small number of serotypes cause most disease today no one knows what will happen in the long run if those serotypes were eliminated due to a vaccine. Replacement with other serotypes may take place not only in carriage as discussed above, but also as cause of invasive disease. These effects can now be seen in the United states, probably because some time has passed since PCV7 was introduced there [120, 122-124]. Serotype switching is also known to occur between pneumococcal strains [25-28], making it possible for virulent clones to "get a new outfit" when their old one is being recognized by the enemy. Finally, the target of a new vaccine would preferably be a protein, since proteins elicit good immune responses in children and the elderly as well as in adults.

1.6 ANTIBIOTIC RESISTANCE

The first clinical pneumococcal isolate that was reported to be resistant to penicillin was isolated from a patient in Australia in 1967 [12]. Since then, penicillin resistance has become a common feature of pneumococci all over the world. Also resistance towards other classes of antibiotics as well as multiresistant (resistant to ≥3 classes of antibiotics) strains have evolved.

1.6.1 Epidemiology of resistance

Pneumococcal drug resistance is increasing worldwide. A good example is penicillin resistance amongst pneumococci in Iceland, a small and easily monitored country. In 1988 the incidence of pneumococcal infections caused by penicillin-resistant strains was 0%. It then increased to 2.3% in 1989, 2.7% in 1990, 8.4% in 1991, 16.3% in 1992, and 19.8% in 1993 [125]. However, the prevalence of resistance varies a lot between different geographical regions. Some Asian countries have reported penicillin resistance in up to 80% of all cases [126], while Sweden has had a constant rate of pneumococci with a reduced susceptibility to penicillin (MIC ≥ 0.5 mg/ml) of about 2% between 1997 and 2003 [127]. It has been shown that resistance is more common within certain serotypes and that much of the resistance can be attributed to a limited number of resistant clones. For example, penicillin resistance is often associated with pneumococci of types 6B, 19F and 23F [126]. A single 6B clone, probably imported from Spain, was shown to be primarily responsible for the 20% increase in penicillin-resistant pneumococci in Iceland discussed above [125].

It is well established that the degree of prescription of antibiotics influences the frequency of resistant bacteria [128, 129]. However, resistance towards erythromycin and trimethoprim/sulfamethoxazole in Sweden was reported to increase between 1997 and 2003, although the prescription and sale of those antibiotics were reduced, showing that other factors are also important [127].

1.6.2 Mechanisms of resistance

Pneumococci can develop resistance via horizontal gene transfer or via point mutations in the genome. It is believed that much of the acquisition of resistance takes place in the nasopharynx. Here, the bacteria reside for a long time and DNA exchange may take place both with other pneumococcal strains as well as with bacteria of other species. Pneumococci do not produce β-lactamases as many other bacteria do. Instead, resistance towards **penicillin** and other β-lactams is mediated by mutated penicillin-binding proteins (PBPs) [130]. Penicillin bind to these proteins, thereby inhibiting the cell wall synthesis and activating the LytA autolysin. The mutated PBPs do not bind penicillin with the same affinity, leading to resistance. This involves a step-wise process with acquisition of more and more mutated PBPs.

Resistance towards most antibiotics beside β-lactams can be obtained either by mutations in the target proteins or by the production of efflux pumps, actively pumping the antibiotics out of the bacteria. This is the case for resistance towards **quinolones** as well as **macrolides.** Quinolones interfere with the bacterial DNA gyrase and topoisomerase IV and point mutations in these proteins can render the bacteria resistant. For the gyrase, this is a two-step process with mutations in the *parC* and *parE* genes, encoding gyrase subunits.

1.6.3 Tolerance

Tolerance was first described in 1970 by Tomasz *et al*. [131]. By definition, a tolerant strain will survive during antibiotic therapy, but does not replicate (figure 5). After removal of the antibiotics, however, growth is resumed. Tolerance is believed to be the platform from

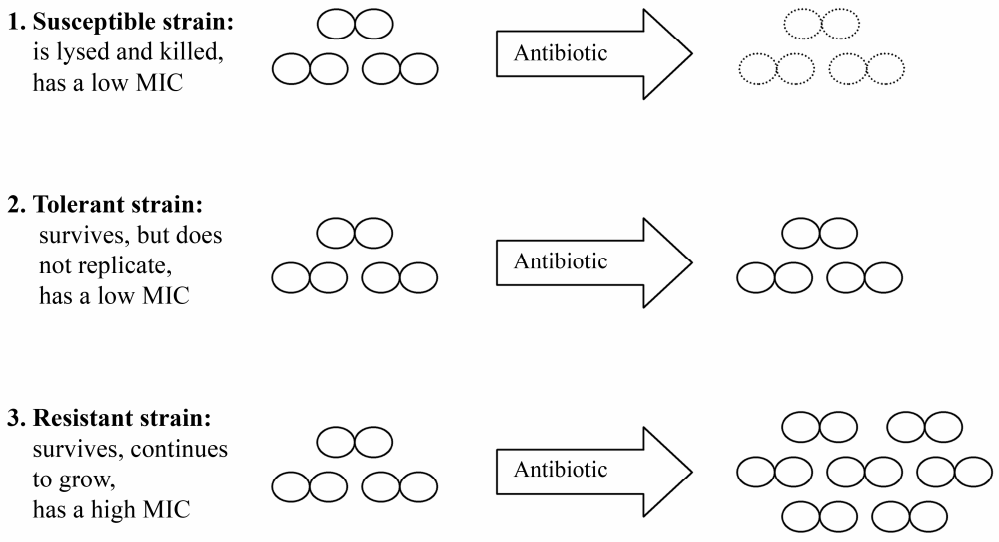


Figure 5. Tolerance and resistance of pneumococcal strains.

which resistant strains can evolve more easily, without loosing to much in fitness. For example, it has been shown that a penicillin-tolerant strain could be transformed to resistant in one single step, which was not the case for the non-tolerant parental strain [132]. This is alarming, since vancomycin-tolerant strains have been reported, that could potentially develop into resistant ones [132, 133].

The molecular background of tolerance is not fully understood. It is important to remember that although deficiency in autolysis generally leads to tolerance, it is not a requirement for tolerance to occur. Models have been proposed, but the exact mechanism remains unknown [132, 134, 135]. Filipe *et al*. have suggested that the *murMN* operon is involved in tolerance [136]. This operon is responsible for branching of the muropeptides of the pneumococcal cell wall and is essential for resistance towards penicillin [137, 138]. Filipe *et al.* showed that a deletion mutant lacking the *murMN* operon was more lytic towards several inhibitors of cell wall synthesis as compared to its parental strain [136]. Also, over-expression of the operon in a penicillin-susceptible strain made the strain tolerant towards penicillin, without changing the MIC (minimum inhibitory concentration).

In the clinical setting tolerance may cause problems since the bacteria are not killed during treatment, but will continue to replicate as soon as the treatment is terminated. Also, it is hard to detect tolerance since tolerant bacteria have the same MIC as susceptible bacteria. The only way to identify tolerant strains is by making a lysis-kill curve [139]. In this assay, a 10×MIC of antibiotic is added to growing pneumococci and the viability is measured 3-4 hours later. A wild-type strain shows at least a 3-log decrease in viability, while a tolerant one shows little or no decline.

1.7 EPIDEMIOLOGY

WHO estimates that 1.6 million people died from pneumococcal disease in 2002. Of these, 712,000 were children under the age of five. This is the largest group of death caused by a vaccine-preventable pathogen [13]. Since 2004, all cases of invasive pneumococcal disease in Sweden are reported to the Swedish Institute for Infectious Disease Control. During 2006 1,334 cases were reported, compared to 1,420 in 2005. The majority (86 %) were isolated from blood.

As described previously, the incidence of pneumococcal carriage is strictly age-associated. This is also the case for pneumococcal disease, with children under 2 years old and the elderly at the highest risk of being affected. It has been proposed that this is due to lack of protective capsule-specific antibodies, since young children cannot elicit the T-cell independent response needed and the elderly have failing immune responses. However, studies have shown that this may not be the full explanation [140].

People at risk, besides the age groups discussed above are those with immuno-deficiencies, both genetic and acquired ones. The incidence of pneumococcal invasive disease is much higher for HIV-infected people than for the rest of the population and they are also at a higher risk of being infected with antibiotic-resistant strains [141-143]. Also those who have undergone splenectomies or have a splenic dysfunction are more susceptible [144]. Infection with influenza virus is another risk factor for pneumococcal disease. Most of the 40-50 million people that died in the 1918 influenza pandemic were killed by secondary bacterial pneumonia [145].

In industrialized countries, indigenous populations have a higher incidence of suffering from pneumococcal disease than the rest of the population. This has been reported in Alaska [146], Australia [147], and the United States [148]. In central Australia, up to 2,000 cases of invasive pneumococcal disease per 100,000 persons have been reported in children under the age of 2, compared to between 10 and 60 per 100,000 for most countries in Western Europe [121].

In recent years, the knowledge of the molecular epidemiology of pneumococcal infections has increased tremendously. Previously, the main focus has been on the capsular type and clearly this is an important virulence factor. Studies have shown that certain serotypes are more commonly involved in certain diseases and for example isolates of types 1, 5 and 7F have a high attack rate, causing less carriage and more invasive disease [34, 149]. Other serotypes such as 19F cause mainly carriage and hence have a low invasive disease potential [34]. Interestingly, the more invasive types have been shown to be genetically highly related, while the carriage types are generally more diverse [34, 150]. Also, recent data from our group suggest that other bacterial factors besides the capsular type might be important for disease outcome and invasive disease potential (unpublished data).

2 AIMS

2.1 PAPER I

To study the correlation between capsular expression and lytic response towards antibiotics. Both the impact of having capsule contra not having capsule and the impact of specific capsular types were investigated.

2.2 PAPER II

To characterize the pneumococcal pathogenicity islet *rlrA* and to show that it is responsible for the production of pneumococcal pili. Also, we investigated the role of pili *in vitro* and *in vivo*.

2.3 PAPER III

To study the spread and mechanisms of success of penicillin nonsusceptible pneumococcal clones.

2.4 PAPER IV

To study the impact of *in vitro* fitness of pneumococcal isolates and mutants on the ability to colonize and cause invasive disease in mice.

3 METHODS

3.1 CHARACTERIZATION OF STRAINS

All isolates included in the studies of this thesis were subjected to **serotyping** and **drug susceptibility testing**. Serotyping was performed with gel diffusion and/or Quelling reaction using type-specific sera obtained from Statens seruminstitut in Copenhagen [151]. Drug susceptibility was determined using the disk diffusion method according to the Swedish Reference Group for Antibiotics (SRGA) with antibiotic discs from Oxoid. Nonsusceptible strains were further analyzed with the E-test (AB Biodisk). In paper I an agar dilution method was used instead [152].

While serotyping is the gold standard when classifying pneumococci, more focus has recently been put on the genetic background of the isolates. Several methods have been developed to obtain genetic footprints of bacterial isolates, making it possible to determine the genetic relatedness of strains. The two methods used in paper III are **pulsed-field gel electrophoresis** (PFGE) and **multilocus sequence typing** (MLST). In PFGE the bacterial genome is cleaved with a restriction enzyme and the fragments are separated on a gel using pulsed electricity. This makes the separation of very large fragments possible, which is not the case for normal gel electrophoresis. The resulting pattern is computer-analyzed and isolates differing with ≤ 3 bands are considered closely related. The PFGE performed in paper III was adapted from Hermans *et al*. and Lefevre *et al*. [153, 154].

MLST is based on the sequencing of parts of seven house-keeping genes in the pneumococcal genome. A large number of alleles have been identified for each of these genes and have been assigned specific numbers. The resulting allelic profile specifies the sequence type (ST) of the analyzed strain. Two isolates are considered to belong to the same clonal cluster if ≤ 2 alleles differ. In relation to PFGE, MLST is less discriminative and is more suitable for long-term studies, while PFGE may be useful for local outbreaks. Also, the interpretation of PFGE result is considered harder and more subjective than that of MLST results, therefore it is easier to compare MLST data between different laboratories.

Microarray was performed in papers III and IV to compare the genomes of clinical isolates. With this high-throughput screening method the presence/absence of genes can be monitored. Oligomers of all genes in the TIGR4 and R6 genomes are spotted onto glass slides and labeled DNA from the test strain is allowed to hybridize to them. Computerized scanning of the slides then allows for the identification of genes present. Limitations of the method are that only the genes included in TIGR4 and R6 are studied and that divergent alleles may be missed due to the use of oligomers.

In paper I we measured the **lysis rates** of pneumococcal isolates, in order to identify strains tolerant to penicillin and/or vancomycin. Antibiotics were added at 10×MIC to bacteria at OD_{620}=0.25 and the OD was monitored for four-five hours; a time that has been shown to differentiate tolerant from non-tolerant strains [155]. Also, viable counts were performed. All assays were carried out without selectable antibiotics to avoid secondary effects as shown for erythromycin by Robertson *et al*. [135].

Finally, in paper IV, we measured the ***in vitro* growth rates** of pneumococcal isolates and mutants. Generation times were determined by measuring OD_{600} of bacteria grown in CY medium at 37°C as a function of time. Bacterial growth was carefully monitored in a Bioscreen equipment, with OD measurements every fifth minute. The growth rates were estimated using the computer program KaleidaGraph™.

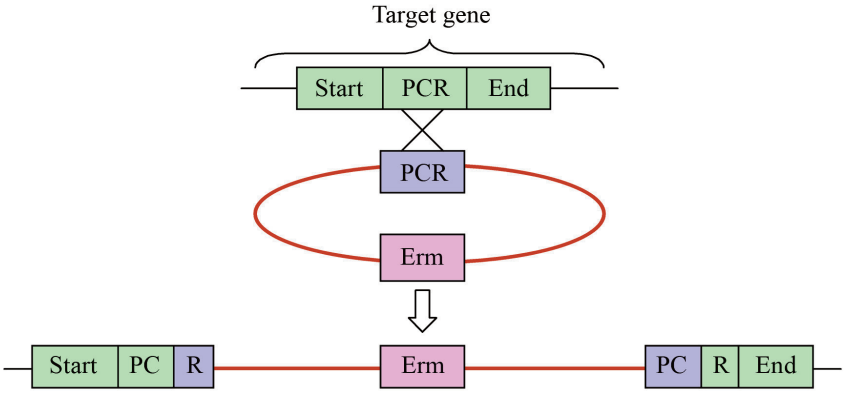
3.2 CREATION OF PNEUMOCOCCAL MUTANTS

Mutants have been created for papers I, II and IV. The methods used for these constructions have become more sophisticated with every study, going from crude insertions of a full plasmid using insertion-duplication mutagenesis, to creation of point mutations using the Janus cassette.

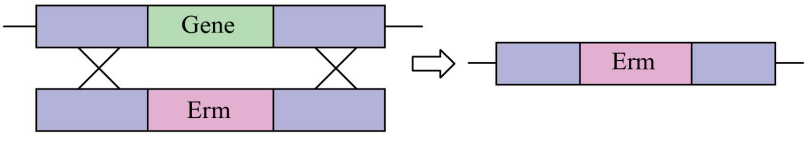
The mutants in paper I were created using **insertion-duplication mutagenesis** (figure 6A). It is a method with a large risk of getting polar effects on genes downstream of the target gene. A fragment containing part of the target gene is constructed and cloned into the pJDC9 vector [156]. After transformation of the recipient strain with this construct, the full vector is inserted into the genome, flanked on both sides by the fragment of the target gene used in the construction (figure 6A). Theoretically this means that the construction is not very stable since cross-over events could easily excise the plasmid. However, this has never been observed in our laboratory.

Insertion-deletion mutagenesis (figure 6B) was used to construct the mutants in papers II and IV. Flanking regions upstream and downstream of the target gene were amplified as well as the erythromycin cassette from pVA838 [157]. The fragments were constructed with *Apa*I and *Bam*HI termini respectively, enabling ligation of upstream and downstream fragments with the erythromycin cassette in between. This linear ligation product was used to transform TIGR4, with selection for Erm^R. This method has been commonly used in pneumococci and is often referred to as PCR ligation mutagenesis [158]. The resulting mutant is stable, but downstream effects may occur. A similar method was used for the **complementation mutant** in paper II. The knocked-out genes were re-introduced into the mutant together with a kanamycin cassette. The kanamycin cassette from Janus [159] was inserted downstream of the target genes in wild-type TIGR4. Chromosomal DNA from that mutant was then used to transform the knockout and restore the wild-type phenotype.

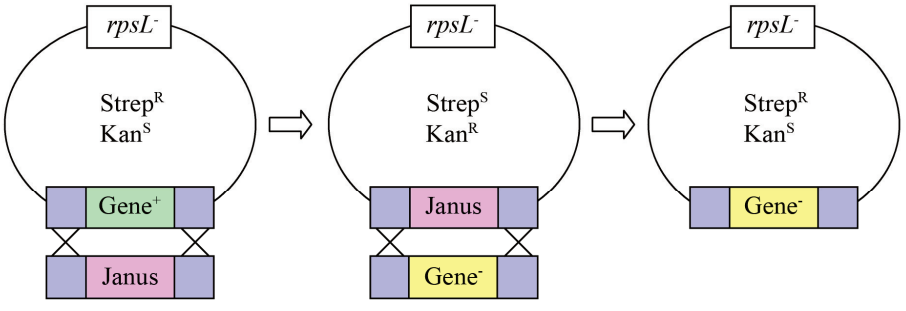
Stop codon mutants were constructed in paper IV (figure 6C). These were produced using the Janus cassette [159] with both positive (kanamycin) and negative (streptomycin) selection. The wild-type strain was a streptomycin-resistant TIGR4 mutant, TIGR4S, containing a substitution (K56→R56) in the *rpsL* gene, previously reported to cause streptomycin resistance [160]. A similar construct as for the insertion-deletion mutants was made, with the Janus cassette surrounded by regions flanking the target gene. This was first transformed into TIGR4 with selection on kanamycin. PCR was run over the full construct, and this fragment in turn was transformed into TIGR4S. Positive clones of TIGR4S (Kan^R Sm^S) were transformed with a PCR product containing the selected stop codons, with selection on streptomycin. Two stop codons were introduced about ten amino acids downstream of the initial ATG. These kinds of mutations rarely give downstream effects and are stable. However, it is much harder to produce mutants with Janus than with insertion-deletion mutagenesis. The reason why we did not transform TIGR4S straight away was that a very high degree of false positives were obtained, showing resistance towards streptomycin as well. The explanation to this, also given by the creators of the Janus cassette [159], seems to be that cross-over occurs between the genomic *rspL* gene (Sm^R) and the one in the cassette (Sm^S). Since the transformation frequency was very low it was difficult to obtain the correct mutants in TIGR4S, which is why we decided to make the construction via TIGR4 (with the wild-type *rspL* gene). By transforming TIGR4 in a first step (not shown in figure 6) and running PCR over the construction, enough material could be produced to get efficient transformation frequencies in TIGR4S.



A. Insertion-duplication mutagenesis



B. Insertion-deletion mutagenesis



C. Point mutations via the Janus cassette

Figure 6. Methods used to construct pneumococcal mutants.

3.3 ANIMAL MODELS

In papers II, III and IV, virulence of different pneumococcal strains was monitored using murine models. This is a common model for pneumococcal disease and has been used in our laboratory with good reproducibility. While infected with our control strain TIGR4, the mice develop disease quite similar to that seen in humans.

The studies in this thesis include **single infections** as well as mixed infections in **competition experiments**. Using competitions, smaller differences in virulence between wild-type and mutant strains can be detected than with single infections. Samples taken from the animals are plated on media with and without antibiotics to calculate the ratio of mutant versus wild-type. The competitive index (CI) is calculated as the ratio of mutant to wild-type output divided by the mutant to wild-type input. In paper III, mixed infections were conducted with two strains lacking antibiotic markers. To calculate the ratio of each strain we analyzed a large number of colonies from each infected mouse using PCR. The PCR was run over the *rlrA* islet, the only locus known to differ between the strains.

Two models have been used in the studies included in this thesis; **intranasal (IN)** and **intraperitoneal (IP)**. The IN model was used to mimic the natural route of transmission, i.e. inhalation of bacterial droplets. A bacterial suspension (5×10^6 CFU in 20 µl) was inoculated intranasally. This is a rather large infectious dose, with a survival rate of about 30% over a week. The animals were monitored for 8 days to assess the health status by clinical scoring. The following scores were used: 0 = healthy, 1 = piloerection, 2 = reduced motility, 3 = more pronounced reduced motility, 4 = 1, 2, 3 more pronounced and 5 = moribund. Mice were sacrificed when they reached score ≥3.

The IP mouse model was mainly used to study inflammation during systemic disease. A bacterial suspension (5×10^6 to 2×10^7 CFU in 200 µl) was inoculated intraperitoneally and the animals were followed over time. All mice were killed at 6 hours post-infection and the serum levels of TNF and IL-6 were determined using commercial ELISA kits.

All animals used in these studies were 5-8 weeks old C57BL/6 mice, either with equal amounts of male and female animals (paper II and III) or only males (paper IV). They were kept with a 12-hours light/dark cycle and had access to standard food and tap water *ad libitum*. The experiments were approved by The Ethical Committee for Animal Experiments in Stockholm and conducted in accordance with the European Communities Council Directive 86/609/EEC.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Capsular expression in *Streptococcus pneumoniae* negatively affects spontaneous and antibiotic-induced lysis and contributes to antibiotic tolerance

This paper aimed at examining the relationship between pneumococcal expression of the polysaccharide capsule and lysis induced spontaneously or by antibiotics. The lytic behavior of clinical isolates is of interest for mainly two reasons. Firstly, LytA-induced lysis is one of the mechanisms by which antibiotics such as penicillin and vancomycin kill pneumococci. Tolerance, a stage known to precede resistance, is characterized by stop in growth, but no lysis or death. Therefore, in order to understand resistance it is crucial to learn more about the factors controlling lysis. Secondly, *in vivo* the lytic response may be of importance for the clinical outcome. When pneumococci lyse, inflammatory particles such as peptidoglycan and teichoic acid are released, which can spread in the body of the host and enhance inflammation. Although the clinical relevance of spontaneous lysis remains to be clarified, the antibiotic-induced lysis is already acknowledged as an important factor for clinical outcome. In the treatment of meningitis, increased inflammation during treatment can be especially devastating [112].

In paper I we show that the capsule may inhibit penicillin- and/or vancomycin-induced lysis of the bacterium, thereby contributing to bacterial tolerance to these antibiotics. Isogenic mutant pairs of encapsulated and nonencapsulated strains in three different serotypic backgrounds were compared for lysis and in all cases the nonencapsulated strains were more lytic than the encapsulated ones. This was true for both antibiotic-induced lysis (figure 7) and spontaneous lysis. However, when deleting *lytA* in the TIGR4 background as well as in the nonencapsulated mutant of this strain, TIGR4R, the LytA-deficient mutants gave a similar lytic response (figure 7A). This indicates that the increased lysis seen in the nonencapsulated strains is LytA-dependant. To further investigate these findings, we studied a mutant, I95R, and its parental strain, I95 (serotype 9V). I95R is a mutant that arose spontaneously in a culture

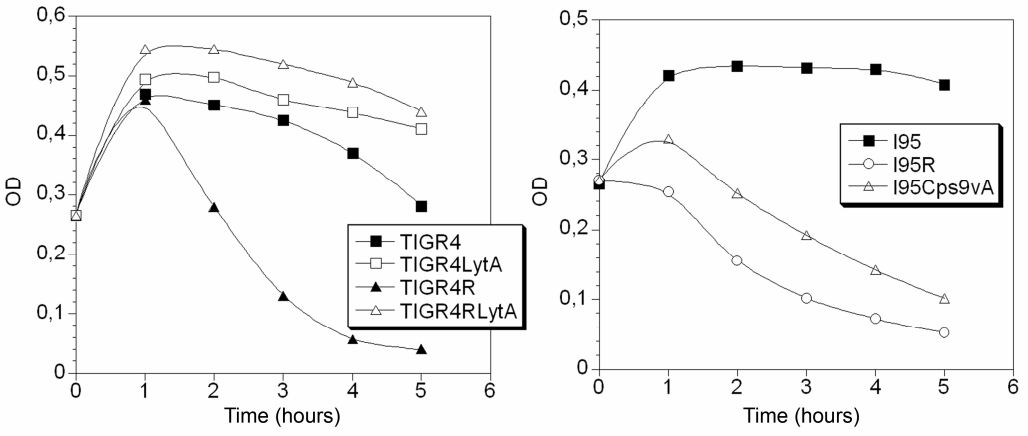


Figure 7. Penicillin-induced lysis of pneumococcal strains. A) TIGR4 (serotype 4) and its non-encapsulated mutant TIGR4R, as well as LytA deletion mutants of both strains. B) I95 (serotype 9V) and its nonencapsulated mutants I95R and 195*Cps9vA*.

when growing I95 (serotype 9V) for several days. I95R was lytic to penicillin and vancomycin as opposed to I95 (figure 7B). By sequencing the capsular operon, we identified the point mutation that gave rise to the loss of capsule. It is a deletion in the fifth gene of the operon, encoding a UDP-1-phosphate transferase. Several other clones of the same kind of mutant were harvested and investigated. All of them contained point mutations in the same gene, some identical to the first one and some different. For some reason these mutants are selected for during prolonged growth, meaning that they have some selective growth advantage, despite the fact that they are more lytic than the parental strain. It is possible that when the supply of nutrients is scarce, it is beneficial to turn down the production of capsule, especially since it is not as important in the *in vitro* setting. This far we have never seen the reverse happening, i.e. the nonencapsulated mutants reverting to the encapsulated phenotype.

Not only did the presence of the capsule have an effect on lysis, the actual type of capsule was also of importance. We studied clinical isolates of several serotypes and found that different serotypes have different impact on lysis. The lytic responses upon addition of 10×MIC of penicillin or vancomycin were compared for isolates of types 1, 3, 4, 6B, 9V, 14 and 23F. Serotype 1, 4, 6B and 23F isolates were generally less lytic towards penicillin than the other isolates. Also, isolates belonging to serotype 9V were the only ones showing reduced lysis towards vancomycin.

It is difficult to explain why capsular expression inhibits lysis. We know that it is not due to differences in expression of LytA, shown by equal amounts of *lytA*-specific transcripts for all strains in Northern blot analysis. Also, using Western blot analysis, the same amount of LytA was detected in all strains. One explanation could be that the capsule sterically blocks LytA from accessing its substrate.

4.2 PAPER II

A pneumococcal pilus influences virulence and host inflammatory responses

Paper II describes a novel pneumococcal virulence factor, the pilus. It has been known previously that the *rlrA* pathogenicity islet is involved in virulence, but the functions and biological roles of its gene products have not been elucidated. We show that the islet is responsible for the production of pneumococcal pili. The islet encodes three proteins – RrgA, RrgB and RrgC – that make up the pilus structure, as well as three sortases. RlrA is a positive regulator of the islet and is also found within the islet. A negative regulator, MgrA, is encoded elsewhere on the chromosome [99], and deleting it results in mutants over-expressing pili (figure 8). Not all pneumococci contain the *rlrA* islet. It is present in TIGR4, but not in R6 [9, 10]. A 19F isolate previously shown by our laboratory to be an excellent colonizer of mice was also shown to harbor the islet.

Several methods were applied to demonstrate that pneumococci harboring the

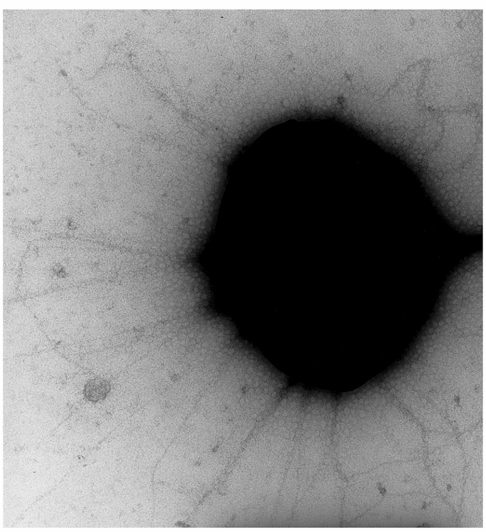


Figure 8. Pili expression in a TIGR4 deletion mutant lacking *mgrA*.

rlrA islet express pili. Mutanolysin-treated pneumococci were separated on a 4-12% polyacrylamide gradient gel and immunoblotted with RrgB-specific antisera. This resulted in a ladder of high molecular weight polymers ranging from <100 kDa to >1,000 kDa, demonstrating the polymeric nature of the pili. Similar patterns have previously been observed for pili in *C. diphteriae* [97]. With EM the pilus polymers could be readily visualized on wild-type TIGR4 as well as on the 19F colonizer isolate. Using immunogold staining we also showed that the RrgA protein is located at the base of the pilus, next to the cell surface. The pilus is mainly built up by RrgB, and RrgC is located at the tip of it. Deletion mutants lacking all three subunits RrgA, RrgB, RrgC and the three sortases were constructed in the TIGR4 background as well as in the 19F background. These strains lacked pilus expression, as shown by both Western blot and EM.

To investigate the *in vivo* role of pili, mouse experiments were conducted. Three types of models were used; intranasal single infections, intranasal competitive infections and intraperitoneal single infections. In the intranasal single infections, mice infected with the TIGR4 mutants lacking pili survived to a higher degree than those infected with the wild-type strain. However, all surviving mice were colonized to a similar extent, independent of infecting strain. To determine if the wild-type could be shown to have an advantage also in colonization, competition experiments were performed. Mice were inoculated intranasally with equal amounts of wild-type and mutant strains. The competitive index (CI) was calculated as the ratio of mutant to wild-type output divided by the mutant to wild-type input. Competitive indices of less than 1 were obtained for colonization, pneumonia and sepsis, indicating that the mutant is attenuated in all these settings. Similar experiments were conducted using the nonpiliated D39 and an *rlrA* islet insertion mutant of this strain and again the islet was shown to provide an advantage in colonization and disease. Finally, the inflammatory response was studied in an IP infection model. Mice were sacrificed 6 hours post-infection and the levels of bacteria, TNF and IL-6 in blood were measured. Mutants lacking pili induced a lower proinflammatory response, suggesting that the pneumococcal pilus is important for inflammation. All animal experiments were also performed using a revertant; the mutant TIGR4 strain into which the *rlrA* islet had been reintroduced. In all cases the wild-type phenotype was restored.

The pneumococcal pilus seems to be an important factor in both colonization, virulence and inflammation. Clones of types 1 and 7F with a high invasive disease potential, shown to cause a mild disease in humans with no lethality [150] were shown to be nonpiliated. These also evoke a low inflammatory response in an IP mouse model [161].

4.3 PAPER III

Clonal success of piliated penicillin nonsusceptible pneumococci

This paper is based on an epidemiological study of pneumococci nonsusceptible to penicillin (PNSP) in Sweden. The dominating serotype amongst PNSP isolates has for a long time been 9V, but lately an increase has been observed for type 14 isolates, making up 12% of PNSP in

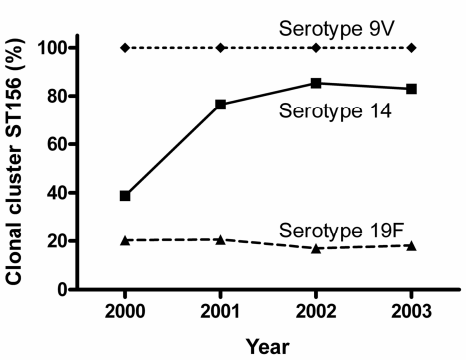


Figure 9. Percentage of the ST156 clonal cluster among pneumococci nonsusceptible to penicillin of types 9V, 14 and 19F in Sweden.

1999 and 26% in 2003. To investigate the molecular background of this increase, we performed molecular typing of 455 type 14 isolates obtained between 2000 and 2003 and compared them with 80 isolates of type 9V from the same time period. PFGE was performed on all isolates, identifying the major clonal clusters for each serotype. A smaller number of isolates, representing all of these major clones were further analyzed with MLST. Interestingly, all of the 9V isolates belonged to the same MLST clone of ST156, an internationally recognized clone known to have been successfully spreading worldwide (figure 9). When looking at the type 14 isolates it turned out that the frequency of ST156 and closely related clones (single-locus variants) in this group had increased from less than 40% in 2000 to more than 80% in 2003 (figure 9). It is therefore likely that a capsular switch occurred in a type 9V ST156 isolate turning it into a type 14 ST156 isolate, giving rise to the increase in type 14 PNSP isolates. The ST156 clonal cluster was also present among the serotype 19F PNSP isolates, but in this group it only made up about 20% of the isolates, a level that was roughly the same between 2000 and 2003 (figure 9). Taken together, these data indicate that about 50% of all PNSP in Sweden in 2003 belonged to the ST156 clonal cluster.

The ST156 clone has been studied in detail for quite some time since it was first reported in Spain in 1988 [25]. It is nonsusceptible to penicillin as well as trimethoprim/ sulfametoxazole and has been isolated on most continents of the world. The successful spread of this clone has previously been attributed to its multiresistance pattern. However, this does not really explain the rapid expansion in Sweden, a country known for its relatively low consumption of antibiotics. Further characterization of the 2000-2003 material with microarray analysis, sequencing and PCR revealed that all but one of the PNSP isolates of types 9V, 14 and 19F included in the ST156 clonal cluster harbored pili. Our group has shown that the pili are important for colonization and virulence in mice, indicating that pili expression may be an important factor for the success of ST156. To further test this theory we studied PNSP isolates of other serotypes than the ones discussed above. Isolates of types 6B and 35B were analyzed with PFGE and MLST, revealing that ST156 was totally absent in these groups. Instead, a number of other clones dominated, for example Spain^{6B}-2 and ST558 (35B). Interestingly, almost all of these clones contained the *rlrA* islet and expressed pili, thereby having the same advantage as ST156. Also, when revisiting the 19F PNSP group, most non-ST156 isolates turned out to belong to a limited number of clones, the major ones all being piliated. We conclude that at least 70% of the Swedish PNSP isolates in 2003 harbored the *rlrA* islet and expressed pili. The success of ST156 amongst some serotypes and not amongst others can be explained by the absence or presence of other piliated clones within these groups.

Microarray analysis of a number of ST156 and non-ST156 isolates revealed a close relationship between the isolates belonging to the same clone. The intra-clonal differences seen amongst these isolates were localized to a 750 kb large fragment of the genome with the

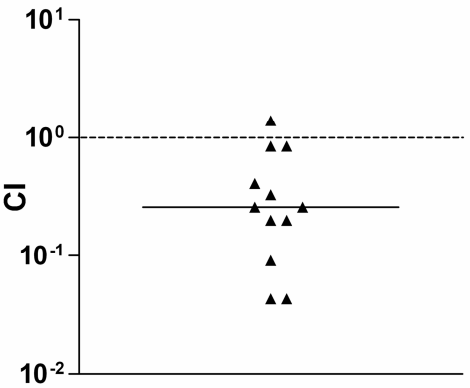


Figure 10. Competition assay with two ST156 isolates of type 19F, one piliated and one non-piliated.

capsular biosynthesis locus in the center, indicating that more than the capsular region was transferred when the capsular switches took place. Microarray also identified two interesting strains that were further studied in an animal model. These strains were both of type 19F and ST156, but one of them lacked the *rlrA* islet (the only nonpiliated ST156 strain in our collection). According to the microarray data this was the only genetic difference between the strains, making them an excellent study pair. We inoculated mice intranasally with the bacteria in equal amounts and observed colonization one week later. The piliated strain had then outcompeted the nonpiliated strain in 75% of the mice, again showing the advantage of piliation in colonization (figure 10). We then looked in our strain collection for serotypes mostly associated with invasive disease and not carriage, such as types 1, 2, 3 and 7F. It turned out that they generally lacked the *rlrA* islet, further establishing the role of pili in colonization.

4.4 PAPER IV

The influence of *in vitro* fitness defects on pneumococcal ability to colonize and to cause invasive disease

Pneumococci are known to cause symptoms in the host by triggering an inflammatory response rather than by generating toxic products such as toxins. Therefore, the ability to grow fast should be a great advantage for pneumococci when it comes to causing disease. This study aimed at investigating just how important the growth rate is for pneumococcal virulence. First, a number of clinical isolates with known disease potential in mice were analyzed for their growth rates. These isolates were of different MLST types and came from different backgrounds, but were showed to have quite similar generation times as monitored with a Bioscreen equipment *in vitro*. Two isolates each of serotypes 1, 6B, 7F, 14 and 19F as well as TIGR4 were studied. Only one isolate, a type 14 carrier isolate, grew significantly slower than the rest, with a generation time of 43 minutes compared to 37 minutes for TIGR4. When comparing the virulence of these isolates pair-wise, the type 14 isolates were the ones differing the most from each other. The slow-growing isolate killed 50% of the mice, while the other type 14 isolate resulted in 90% killing. Microarray analysis was performed on the two type 14 isolates, trying to find out what was behind the defect in growth and virulence. However, these isolates were too divergent to pinpoint the exact mechanism of attenuation.

To further investigate the relationship between *in vitro* fitness and *in vivo* virulence, a panel of mutants were constructed in the TIGR4 background. Five genes were chosen, based on a study by Garbom *et al*. [162], representing non-essential house-keeping genes likely to give growth defects when knocked-out. The genes, *ychF*, *smf*, *yebC*, *rluD* and *hemK*, were successfully knocked out via insertion-deletion mutagenesis. This method may give polar effects on down-stream genes, but since we were mainly interested in creating growth-defect mutants this did not matter. Generation times were measured for all mutants and the *rluD* and *smf* mutants showed almost wild-type fitness with generation times of 39 and 36 minutes respectively, as compared to 37 minutes for the wild-type TIGR4 strain ($p=0.0596$ and $p=0.4452$). However, the insertion-deletion mutants of *ychF*, *hemK* and *yebC* all had reduced growth rates, 42, 65 and 48 minutes respectively ($p=0.0003$, $p<0.0001$ and $p<0.0001$). The growth defect was especially pronounced for the *hemK* mutant, growing with smaller colonies on blood agar plates. Mice were then challenged intranasally with the mutants. All mutants gave rise to the same type of disease, but with different frequencies. For those mice where bacteria could be cultured from blood, the health status rapidly and irreversibly deteriorated. The three mutants attenuated in growth, TIGR4Δ*ychF*, TIGR4Δ*hemK* and TIGR4Δ*yebC,* gave a higher end-point survival than the wild-type TIGR4

strain with 55% (χ^2=10.25; p=0.0014), 77% (χ^2=18.41; p<0.0001) and 56% (χ^2=7.369; p=0.0066) survival respectively by 192 hours, compared to 31% for TIGR4. In contrast, TIGR4Δ*rluD*, and TIGR4Δ*smf*, were not attenuated, with survival rates of 38% (χ^2=0.3529; p=0.5525) and 32% (χ^2=0.0002264; p=0.9880) respectively. Thus, the growth defect pattern matched the virulence pattern very well for these mutants. However, the growth-attenuated mutants were all capable of colonizing the mice to a similar extent 8 days post infection as TIGR4.

Since the HemK mutant was so severely attenuated in both growth and virulence we wanted to study it further. In *E. coli hemK* encodes an *N*(5)-glutamine methyltransferase that modifies peptide release factors [163]. In *S. pneumoniae hemK* is the fourth gene out of eleven in an operon that has previously been reported to be associated with virulence [16, 17]. Two groups have reported the effects of mutations in *hemK* or the *hemK* operon in *S. pneumoniae*. While Marra *et al*. observed a significant effect on virulence, Orihuela *et al*. did not observe any attenuation [16, 17]. In order to further study this operon, we constructed mutants containing stop codons in the beginning of the target genes. We targeted *hemK* and the two genes directly downstream of it, SP1022 and SP1023. Two stops were introduced into each gene about ten amino acids downstream of the start codon. Only one of these mutants, the one in SP1022, grew with the same kind of small colonies as the *hemK* insertion-deletion mutant and this mutant was also attenuated in virulence. The homologue to SP1022 in *E. coli*, *yrdC*, encodes a protein responsible for ribosomal maturation and knocking it out leads to growth defects [164]. It would not be surprising if the pneumococcal variant has a similar function.

In conclusion, small variations in growth rate can influence virulence. These variations may not be revealed in a growth curve, instead the growth rate should be accurately calculated.

5 CONCLUDING REMARKS

Much of the work in this thesis has dealt with pneumococcal virulence. I believe that each pneumococcal strain is equipped with a unique combination of factors that are needed for that specific strain to cause disease. It will be a combination of capsular type and presence of known as well as unknown virulence factors that determines a strains ability to cause disease. In order to identify general, universal pneumococcal virulence factors, one has to study a panel of strains, of both different serotypes and different clonalities. Also, I think that examining clinical isolates is crucial to determine the clinical relevance of new findings. A novel virulence factor only present in a small subset of clinical strains is simply not as interesting as a highly prevalent one. For me, it is not surprising that the large screening studies that have been performed to identify features important for virulence often result in a gene content that is not overlapping with other similar studies. These studies typically look at only one pneumococcal isolate, of one capsular and clonal type. The proportion of genes shown to be of importance for virulence is also doubtfully large. It is not surprising that many genes are essential for virulence, but are they essential *only* for virulence? A virulence factor should be something that is needed for virulence, but not necessarily for bacterial survival outside of the host. It is of course hard to know where to draw the line and many traits may end up somewhere in-between. However, one needs to be more critical before saying that something is a virulence factor. The work in paper IV shows that even small changes in growth rate may affect virulence. These changes are so small that they may be hard to detect just by making a growth curve.

When it comes to my work regarding pneumococcal lysis and its correlation to capsular expression, it would be interesting to make a follow-up study. Here, I would like to construct capsule switch mutants and test the lytic response of those strains. Investigating antibiotic-induced lysis in a panel of mutants with the same genetic backgrounds, but different capsules would make it possible to directly see the impact that capsular type has on lysis and tolerance. Furthermore, it would be interesting to have a second look at the strains that we used in paper I. These were chosen to be as different as possible, by taking strains isolated at different time points, at different geographical locations and from different body compartments. Still, we do not know the exact genetic background of those isolates, hence they may be very similar or diverse. It is highly unlikely that each of the serotypes studied would represent a single clone, but it is possible. The work in paper III has taught us that studying the molecular epidemiology of pneumococcal isolates may open up for new explanations of studied phenomena.

Finally, how should we combat pneumococci in the future? Even if a vaccine would be efficient towards the most commonly found serotypes, I would be very surprised if other serotypes would not replace them. For pneumococci it is not as straight-forward as for *Haemophilus influenzae*, where the more serious infections were almost exclusively caused by type B strains. Instead, most (if not all) serotypes are cable of causing life-threatening disease. In my opinion a vaccine directed towards non-capsular targets is the only solution. Several vaccine candidates have been proposed and one, the pilus, is currently under investigation in our laboratory. Intranasal vaccination with the RrgB subunit has shown good protection in mice (submitted data). As for many other protein candidates, the pilus has the disadvantage that it is not expressed by all strains. The ultimate solution would be a cocktail including several proteins, thereby covering most strains.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Pneumokocker är bakterier som kan orsaka allvarliga sjukdomar såsom lunginflammation, hjärnhinneinflammation och blodförgiftning, men även mindre farliga sjukdomar som öroninflammation. Det är mycket vanligt att friska personer, främst barn, fungerar som bärare av pneumokocker. Bakterierna *koloniserar* då *nasopharynx*, det vill säga de sitter bundna till celler i bakre näsväggen/svalget, utan att göra personen någon skada. Man vet inte exakt vad som orsakar övergången från det ofarliga bärarstadiet till sjukdom, men även en bärare kan smitta någon annan så att den personen blir sjuk.

Infektioner med pneumokocker har historiskt sett alltid behandlats med antibiotika, till exempel penicillin, men under de senaste årtiondena har resistenta pneumokocker blivit allt vanligare. *Resistens* innebär att bakterierna har förändrats så att antibiotikan inte längre biter på dem. I stora delar av världen har detta blivit ett problem, men i Sverige är resistens än så länge relativt ovanligt. När man dödar pneumokocker med antibiotika gör det så att cellerna spricker, *lyserar*. Mekanismen bakom detta är inte helt utredd, men man vet att ett enzym hos bakterierna, *LytA*, aktiveras. Detta får sedan cellerna att lysera, spricka. Den **första artikeln** i min avhandling handlar om just lys och vad som kontrollerar lys. Nästan alla pneumokocker har en *kapsel*, ett skal, av sockermolekyler som har visat sig nödvändig för att bakterierna ska kunna orsaka sjukdom. Dessa kapslar finns av olika sorter, och baserat på vilken sorts kapsel en viss bakterie har så delar man in dem i olika *serotyper*. I artikel I visar vi att bakterier med olika kapslar, av olika serotyper, reagerar olika då man tillsätter antibiotika. Vi testade både penicillin och vancomycin och det visade sig att bakterier av serotyp 1, 4, 6B and 23F lyserade mindre vid penicillintillsats än bakterier av andra typer. Vidare var de bakterier som hade kapsel av typ 9V mindre lytiska efter vancomycintillsats än de andra bakterierna. Vi kunde också visa att om man tog bort kapseln helt, det vill säga konstruerade mutanter utan kapsel, så blev de mycket mer lytiska än de som hade kapsel.

Min forskning har även syftat till att ta reda på mer om vilka egenskaper hos pneumokockerna som gör så att de kan orsaka sjukdom. Man talar ofta om *virulens*, förmågan att ge sjukdom, samt om *virulensfaktorer*, egenskaper hos bakterierna som möjliggör virulensen. Tre av artiklarna i den här avhandlingen handlar främst om virulens, artikel II, III och IV. I **artikel II** beskriver vi en ny virulensfaktor hos pneumokocker, den så kallade *pilusstrukturen*. *Pili* är molekyler som finns på ytan hos många olika bakterier och ofta är av betydelse för virulensen. De ser ut som långa känselspröt som sticker ut från cellen och är i regel uppbyggda av flera byggstenar, olika sorters proteiner. Genom att fotografera pneumokocker med elektronmikroskop som förstorar 40 miljoner gånger har vi nu visat att pneumokocker också har pili. Man kan märka in de olika proteinerna med små kulor som syns i elektronmikroskopet och genom att göra det har vi kunnat visa att pilusstrukturen är uppbyggd av tre olika sorters byggstenar, RrgA, RrgB och RrgC. Merparten av pili består av RrgB, med RrgA närmast cellytan och RrgC i toppen av den långa strukturen. Vi har konstruerat pneumokocker utan pili, mutanter, som vi sedan har studerat i en djurmodell. Det har då visat sig att mutanter utan pili är sämre på att kolonisera möss och även ger mindre sjukdom.

I **artikel III** studerar vi epidemiologiskt vilka pneumokocker med nedsatt känslighet för penicillin (*PNSP*) som varit vanligast i Sverige under ett antal år. Vi tittar då på både utsidan och insidan av bakterierna, med utsidan representerad av serotypen (kapseln) och insidan representerad av *arvsanlaget*, *DNA:t*. DNA kan studeras på en mängd olika sätt och

en metod, *MLST* (multilocus sequence typing), är att jämföra sju gener som finns hos alla pneumokocker. Dessa gener finns i ett begränsat antal varianter, så genom att bestämma vilken variant av varje gen som en bakterie har så får den en viss *genetisk profil*, *ST* (sequence type). Bakterier med samma ST är genetiskt besläktade och man talar om att de tillhör samma *klon*. Mellan åren 2000 och 2003 blev PNSP av serotyp 14 allt vanligare i Sverige. När vi tittade på vilka ST som dessa bakterier hade visade det sig att de flesta tillhörde samma klon, ST156. Denna ST har varit och är väldigt vanlig bland serotyp 9V-stammar, varför vi tror att det har skett ett kapselbyte hos klonen. Detta kan ske om bakterier av en viss kapseltyp plockar upp DNA-bitar från bakterier av en annan serotyp och börjar göra kapsel utifrån det nya DNA:t. Vi tror alltså att en klon av typ 9V har plockat upp DNA från en typ 14-stam och därmed börjat producera kapsel av typ 14. Insidan är således densamma som tidigare, ST156, men utsidan har förändrats och blivit typ 14 istället. Vidare undrar vi varför just denna klonen lyckats sprida sig så bra i hela världen, och konstaterar att den har pili, en viktig faktor för både kolonisering och virulens.

Den **fjärde artikeln** handlar om bakteriell *tillväxthastighet*. Vi tittar här på vilken betydelse olika pneumokockers tillväxt-hastighet har för deras förmåga att orsaka sjukdom. Bakterierna har då odlats *in vitro*, det vill säga i ett provrör, och sedan jämför vi deras tillväxthastighet med deras virulens *in vivo*, det vill säga i djurmodeller. Bland de bakterier som vi studerat finns bland annat mutanter, i vilka vi har tagit bort vissa proteiner. Det visar sig att så fort förändringen innebär en minskad förmåga att växa så minskar också virulensen när man infekterar möss med mutanterna. Pneumokocker tillverkar inte lika mycket giftiga ämnen som många andra bakterier, så deras förmåga att orsaka sjukdom beror till stor del på förmågan att växa inuti människan. Flera stora studier har genomförts för att försöka identifiera nya virulensfaktorer hos pneumokocker. Man har då producerat mutanter vilka man testat i djurmodeller, på samma sätt som vi gjort. Dock saknas det ofta uppgifter om tillväxthastigheten hos mutanterna, varför man kan misstänka att många av de identifierade virulensfaktorerna inte är ”sanna” virulensfaktorer, utan snarare proteiner som bakterien behöver för att kunna växa normalt.

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