

Streptococcus pneumoniae

the role of HexA and SpxB in genetic plasticity, and the influence of the capsule genes on the serotype-specific biology

Interfakultäre Inauguraldissertation
der Philosophisch-naturwissenschaftlichen und der Medizinischen Fakultät
der Universität Bern

vorgelegt von

Patrick Kurt Bättig

von Grosswangen, LU

Arbeit unter der Leitung von:

Prof. Dr. med. Kathrin Mühlemann
Institut für Infektionskrankheiten der Universität Bern

und

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Von der Philosophisch-naturwissenschaftlichen und der Medizinischen
Fakultät auf Antrag der Interfakultären Kommission angenommen.

Bern,

Der Dekan der Phil.-nat. Fakultät

Der Dekan der Medizinischen Fakultät

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List of abbreviations

AOM	Acute otitis media
BHI	Brain heart infusion
CCR	Carbon catabolite repression
CFU	Colony forming unit
CSBA	Columbia sheep blood agar
CSP	Competence stimulating peptide
Cm ^R	Chloramphenicol-resistant
Cm ^S	Chloramphenicol-sensitive
DNA	Deoxyribonucleic acid
dTDP	Deoxythymidine diphosphate
Em ^R	Erythromycin-resistant
Em ^S	Erythromycin-sensitive
FCS	Fetal calf serum
Glc	Glucose
GlcA	Glucuronic acid
H ₂ O ₂	Hydrogen peroxide
MIC	Minimal inhibitory concentration
MLST	Multilocus sequence typing
MMR	Mismatch repair system
OD ₆₀₀	Optical density at 600 nanometers
PCV7	7-valent conjugated, pneumococcal polysaccharide vaccine
PFGE	Pulsed-field gelelectrophoresis
Rif ^R	Rifampicin-resistant
RmlA	Glucose-1-phosphate thymidyltransferase
RmlB	dTDP-D-glucose 4,6-dehydratase
RmlC	dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase
RmlD	dTDP-6-deoxy-L-xylo-4-hexulose reductase
Sm ^R	Streptomycin-resistant
TMP/SMX	Trimethoprim-sulfamethoxazole
THY	Todd Hewitt with yeast extract
UDP	Uridine diphosphate
Ugd	UDP-glucose-dehydrogenase

Overview and summary of the thesis

The manuscript of this thesis is organized in seven chapters.

First, **chapter 1** provides the aims of this thesis. It then gives an overview of the importance of *S. pneumoniae* as a human pathogen and new threats due to the emergence of antibiotic resistance and vaccine escape mutants. The next two topics outline the background for the main topics of this thesis: mechanisms of genetic plasticity in *S. pneumoniae* and the biology of pneumococcal polysaccharide capsules.

The purpose of the experiments described in **chapter 2** was to investigate the role of mismatch repair gene *hexA* in the mutator phenotype observed in Swiss clinical pneumococcal isolates. It was shown that the mutator phenotype is associated with certain clones, which include a non-typable clone spreading around the globe. Serious technical difficulties were encountered when complementing a *hexA* knock-out recipient with *hexA* from a mutator strain. This chapter summarizes the experiments which were performed so far, discusses the problems encountered and presents a strategy to overcome the technical problems.

Chapter 3 describes the investigation of two clinical pneumococcal isolates with high mutation rate to optochin resistance, but with normal mutation rate to rifampicin resistance. It could be shown that this mutator phenotype was associated with an increased production of hydrogen peroxide (H₂O₂) in the early growth phase due to overexpression of the gene *spxB* encoding the pyruvate oxidase.

Experiments described in **Chapter 4** demonstrate that pneumococcal *spxB* gene plays a role in the competence associated DNA release from a fraction of bacterial cells. No firm evidence could be found for our hypothesis that DNA release was mediated by a toxic effect of H₂O₂. Also, neither of the two products of SpxB, H₂O₂ and acetyl phosphate, seemed to act as intracellular messengers for DNA release. Interestingly, deletion of *spxB* reduced the transformability to undetectable levels. A link between *spxB* and competence is currently being investigated in gene expression studies of the competence genes *recA* and *comC*.

Pneumococcal serotypes differ for invasive potential and colonization prevalence for unknown reasons. In **chapter 5** the in vitro growth of 15 different serotypes is compared. It was found, that the length of the lag phase differed between serotypes and was longer in serotypes associated with invasive disease and shorter in serotypes with an increased propensity for colonization.

Based on the serotype-specific growth behavior described in chapter 5, **chapter 6** investigated whether the in vitro growth of pneumococcal strain D39 (serotype 2) is influenced by its capsule genes. Deletion of all pneumococcal capsule genes induced a prolonged lag phase. Partial deletion of the capsule operon showed different effects on the lag phase. Therefore, observed phenomenon could not be ascribed to a single capsule gene. Interestingly, supplementation with fetal calf serum compensated the prolonged lag phase of capsule gene-deficient mutants.

Chapter 7 includes the annex, the curriculum vitae and the references.

The annex shows a publication to which I contributed. In this paper the capsule region of non-encapsulated Swiss clinical isolates were investigated. Two groups were identified based on the structure of the capsule region and MLST. Group I was closely related to encapsulated pneumococci. Group II was found to carry genes with homology to the *aliB* gene in the capsule region.

CHAPTER 1

Introduction

- 1. Aims of this thesis**
- 2. The importance of *S. pneumoniae* infection and new threats**
- 3. Mechanisms of genetic plasticity in *S. pneumoniae***
- 4. The polysaccharide capsule of *S. pneumoniae***

Introduction

1. Aims of this thesis

In this thesis two main areas were of interest.

Firstly, new problems with *S. pneumoniae* such as antibiotic resistance and vaccine escape mutants rely on the genetic plasticity of this bacterium. In this work two mechanisms of genetic evolution were studied in more detail:

A) The role of the mismatch repair system HexA for the mutator phenotype

B) The role of SpxB, i.e. its product, hydrogen peroxide, on:

B1) the mutator phenotype

B2) DNA release during competence

Secondly, *S. pneumoniae* serotypes exhibit their own distinct biology and epidemiology. New pneumococcal vaccines include only a small selection of pneumococcal serotypes and vaccine pressure induces serotype redistribution. The factors that favor the selection of some serotypes, but not others, (with or without vaccine selection pressure) are not understood. In this work the hypothesis tested is whether

C) The serotype-specific capsule genes may influence pneumococcal growth and, therefore, act as a fitness factor

2. The importance of *Streptococcus pneumoniae* infection and new threats

A. PNEUMOCOCCUS BELONGS TO THE MOST IMPORTANT HUMAN PATHOGENS: *Streptococcus pneumoniae* is currently the most frequent cause of bacterial meningitis, community acquired bacterial pneumonia and acute otitis media. Pneumococcal infections predominate among children <5 years of age, the elderly >64 years of age and patients with suppressed immunity. In Switzerland, the annual incidence of reported invasive pneumococcal infections is: 10 / 100'000 overall, 19 / 100'000 during the first year of life and 34 / 100'000 during the second year of life [1]. However, true infection rates are likely even higher, since oftentimes pneumococcal pneumonia and bacteremia escape surveillance due to the lack of microbiological confirmation of the diagnosis. Acute otitis media (AOM) caused by *S. pneumoniae* is responsible for approximately 68'000 first outpatient visits per year in Switzerland [2]. AOM is also one of the most common reasons for antibiotic prescription in the outpatient setting in industrialized countries [3].

B. MORBIDITY AND LETHALITY OF PNEUMOCOCCAL INFECTION REMAINS HIGH: Despite the availability of antibiotics lethality of invasive pneumococcal infections remains high. In Switzerland, the overall lethality of invasive pneumococcal infections is 11%, 9% during the first year of life and 15% among the elderly >64 years of age [1, 4, 5]. It has been estimated, that approximately 25% of patients with pneumococcal meningitis will have longterm sequelae [6].

C. THERAPY OF PNEUMOCOCCAL INFECTIONS IS THREATENED BY INCREASING ANTIBIOTIC RESISTANCE: During the past decades, antibiotic resistance in *S. pneumoniae* isolates has emerged and spread rapidly world-wide. Resistance rates exhibit considerable geographical variability [7]. In Switzerland, overall penicillin resistance rates have remained relatively low (10%, of which 2% are high-resistant) [1, 4]. Higher rates can be observed in risk groups, such as children <2 years of age (up to 25%), and in geographical regions, such as Western Switzerland (17%) [1, 4, 8, 9]. Current resistance rates in Switzerland already

determine the choice of empirical therapy for severe pneumococcal infection, such as meningitis.

D. SUCCESS OF VACCINATION MAY BE HAMPERED BY ESCAPE MUTANTS:

The new 7-valent conjugated vaccine (PCV7) is highly efficient in reducing the morbidity and mortality of invasive pneumococcal infections in young children [10]. In the USA the incidence of invasive pneumococcal infections has been reduced by >80% since the introduction of universal vaccination of children with PCV7 in 2000 [11, 12]. Herdimmunity mediated by the reduction of nasopharyngeal colonization in vaccinated children has also led to a significant reduction of disease incidence among adult high risk groups, such as the elderly and persons with HIV [3, 11, 13]. Broad administration of PCV has also reduced the prevalence of pneumococcal resistance, since antibiotic resistance is strongly associated with pneumococcal serotypes included in the vaccine [12, 14-16].

In Switzerland, PCV7 has been licensed in 2000 and has initially been recommended for high risk groups [17]. Recently, the recommendation has been extended to all children <5 years of age [18].

Vaccine pressure may select for new virulent pneumococcal serotypes and hamper vaccine effectiveness: PCV7 covers only 7 of the 90 pneumococcal serotypes. Due to the selection pressure of the vaccine exerted on the nasopharyngeal mucosa, non-vaccine serotypes and new pneumococcal serotypes may emerge. Already during the early vaccine trials, serotype replacement by non-vaccine types among colonizing pneumococci has been observed [19-21]. Postlicensure studies confirm a shift of colonizing serotypes towards non-vaccine serotypes [22, 23]. Emergence of non-vaccine serotypes 3 and 19A among invasive isolates have recently reported [11, 13, 14].

The mechanisms of serotype replacement and emergence of new serotypes are poorly understood: Variation of serotype distribution over time and between geographic regions and populations has been observed also before the introduction of PCV7 [24-26]. For example, a) the pediatric serotypes (6B, 9V, 14, 19F, 23F) occur more frequently among young children and the elderly [27], b) the relative proportion of serotypes covered by PCV7 has increased significantly since the beginning of the 20th century [28], and c) epidemic increases of serotypes, such as serotype 1, have been reported [14, 29, 30]. Several factors have been proposed to

play a role for the dynamics of the serotype epidemiology, such as a) altered immunity at young or old age or due to comorbidities, b) sociodemographic factors (crowding, day care), c) antibiotic selection pressure [8, 25, 27], and d) bacterial factors that remain to be characterized.

3. Mechanisms of genetic plasticity in *S. pneumoniae*

A. THE GENETIC DIVERSITY OF PNEUMOCOCCUS IS MAINLY DUE TO HORIZONTAL GENE TRANSFER.

S. pneumoniae is naturally transformable and horizontal gene transfer by transformation [31-33] is the most important mechanism for genetic evolution of pneumococcal populations. Conjugation and transduction seem to be relatively unimportant [34]. Also Feil et al. [35] showed that transformation is significantly more important for the evolution of *S. pneumoniae* than spontaneous mutational events.

The status at which pneumococcal cells have the ability to take up foreign DNA is called competence (for a review see [36]). Competence is induced by a small peptide pheromone called CSP (competence stimulating peptide). A two-component regulatory system detects CSP and transmits a signal that leads to transcriptional activation of the competence regulon (com regulon). The com regulon encodes for multiple genes and induces in complex pathways the machinery for DNA uptake and its processing. Competence is tightly regulated and is maintained for about 15 minutes after the induction with CSP and then decays almost as quickly as it arose.

Competence induces cell lysis and DNA release from a subfraction of the cell population:

The competence regulon serves purposes other than genetic transformation. Havarstein et al. and Claverys et al. discovered, that competence triggers cell lysis and DNA release from a subpopulation of bacterial cells [37-39]. Competence-triggered DNA release enhances the genetic plasticity due to coordination in time and space between release and uptake of DNA by competent cells. Cell lysis and subsequent DNA release requires at least one bacteriocin system (*cibABC*) and three cell-wall hydrolases (*cbpD*, *lytA*, and *lytC*) [37-41]. However, these genes can not account for all of the DNA liberation.

In this thesis it was investigated whether endogenous H₂O₂, is involved in DNA release, possibly by direct toxic effect on the cells (mechanism of H₂O₂ production is described below).

B. MUTATOR PHENOTYPES ALLOW BACTERIA TO ADAPT RAPIDLY TO CHANGING ENVIRONMENTS.

Although horizontal gene transfer determines the evolution of *S. pneumoniae*, spontaneous mutation also plays some role. Recently, mutator phenotypes have found increased interest also in bacteria [42-44]. In

pneumococci, spontaneous mutations occur at a frequency of approximately 10^{-8} , whereas mutators exhibit a mutation frequency of 10^{-7} or higher [45]. In *S. pneumoniae* mutator phenotypes have been described to be the result of inactivation of the HexAB system [45, 46] or of an atypical *mutY* homologue [47] (Table 1).

Table 1. Bacterial systems which can induce a mutator phenotype

Involved bacterial system	Enzyme*	Mutator phenotype induced by	Induced base changes	Reference
Mismatch repair system	HexA ^a , HexB ^a	Mutations	A → G and G → A	[45, 48-51]
Hydrogen peroxide	pyruvate oxidase ^a	↑ H ₂ O ₂ release?	G → A > G → T	[52, 53]
GO [#] system	MutY ^{a,b} , MutM ^b , MutT ^b	Mutations	G → T [§]	[47, 54, 55]
Replicative DNA polymerases	ε subunit of DNA polymerase III ^b	Mutations	All base substitutions	[54, 56]
Specialized DNA polymerases	Pol IV ^b , V ^b , II ^b	↑ expression	Situation dependent ^ε	[42, 44, 54, 57]

* enzymes identified in *S. pneumoniae* (a) or *Escherichia coli* (b) with hypothetical function in *S. pneumoniae*

[#] 8-oxodG, system prevents mutations caused by oxidized guanine base

[§] typical base change by MutY deficiency

^ε e.g. repairing thymine-thymine dimers induced during UV exposure

B.1. Deficiency in the pneumococcal mismatch repair (MMR) system HexAB showed a 10-fold increase of the spontaneous mutation rate [51, 58, 59]. Two *hex* genes, *hexA* and *hexB*, have been identified in *S. pneumoniae* [60]. The Hex system is able to recognize noncomplementary base pairs in DNA. It acts after DNA replication to remove misincorporated bases. Different base-base mismatches are repaired by the Hex system, but with different efficiencies. Transition mismatches (i.e G/T and A/C) are preferentially recognized and repaired [48]. HexA (homologue to MutS in *E. coli*) recognizes the mismatched base pair, but the role of HexB has not been demonstrated. By analogy with its *E. coli* homologue, it is likely to interact with HexA and other proteins in the mismatch repair complex and stimulate their activities. For example, excision and replacement of the noncomplementary base [54, 61]. How does the Hex system recognize the newly synthesized daughter strand during replication or the donor DNA during transformation from the parental strand? Unlike the Mut system in *E. coli*, the Hex system does not require recognition of unmethylated sequences in hemimethylated DNA. In *S. pneumoniae* the daughter strand as well as the donor DNA during recombination are recognized by single-

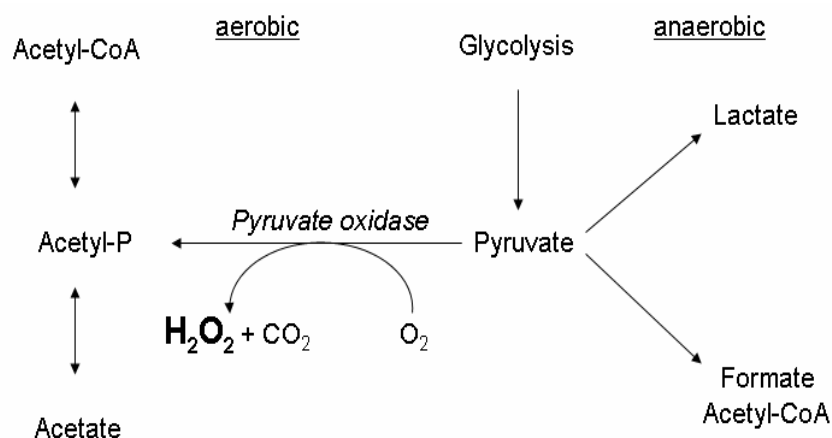
strand breaks [48]. For the donor fragment, breaks are found at both ends before incorporation. During replication, breaks are constantly produced, because the daughter strand is synthesized as short fragments (Okazaki fragments) on the lagging strand [48].

The Hex system influences also the transformation rate. MMR systems can be potent inhibitors of recombination between related species [62]. In *S. pneumoniae*, the generalized MMR system Hex can reduce transformation frequencies for point mutations in homologous DNA up to 20-fold by repairing mismatches in the incoming DNA. However, the Hex system is unable to prevent interspecies transformation due to saturation by an excess of mismatches [51]. This is also supported by the fact that the Hex system is not induced during transformation [51].

B.2. Hydrogen peroxide causes oxidative DNA damage. *S. pneumoniae* generates substantial amounts of H_2O_2 , up to 1-2 mM under aerobic and rich growth conditions [52, 63]. Hydrogen peroxide is produced by the enzyme pyruvate oxidase (Fig. 1, [64]). Pneumococcal *spxB* gene encodes for the pyruvate oxidase.

Pericone et al. showed that H_2O_2 increases the mutation rate to optochin [52]. Hydrogen peroxide causes oxidative DNA damage through hydroxyl radicals produced via the Fenton reaction [65, 66]: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^\bullet + OH^-$. Guanine bases are particularly susceptible to oxygen radicals. Oxidized guanine base (8-oxodG) introduces point mutations by pairing with adenine or thymine, instead of cytosine [53].

Figure 1. Production of H_2O_2 in *Streptococcus pneumoniae*



B.3. A three-component system (MutY, MutM and MutT in *E. coli*) prevents mutations caused by the oxidative lesion 8-oxodG, or the “GO” lesion. In *E. coli*, MutM and MutY remove oxidized guanine base across from C and A, respectively in double-stranded DNA. In addition, MutY removes also the A from a G:A mispair [54, 67-71]. MutT converts 8-oxodGTP to 8-oxodGMP and prevents therefore the incorporation of the GO lesion into the DNA [72].

B.4. DNA polymerases: Replicative DNA polymerases synthesize quite faithfully, due to their proofreading function (ϵ subunit of DNA polymerase III in *E. coli*) by editing out incorrectly paired bases [73-76]. Deficient proofreading function leads to a mutator phenotype. The mutations that occur include all base substitutions [54].

Specialized DNA polymerases (Pol IV, Pol V and Pol II in *E. coli*) are induced during an SOS response (a response to DNA damage, i.e. UV exposure). Their primary function apparently is to repair damaged DNA. However, these enzymes can also introduce mutations into undamaged DNA. A mutator phenotype can be induced if there is an overexpression of specialized DNA polymerases under normal conditions [42, 54].

4. The polysaccharide capsule of *S. pneumoniae*

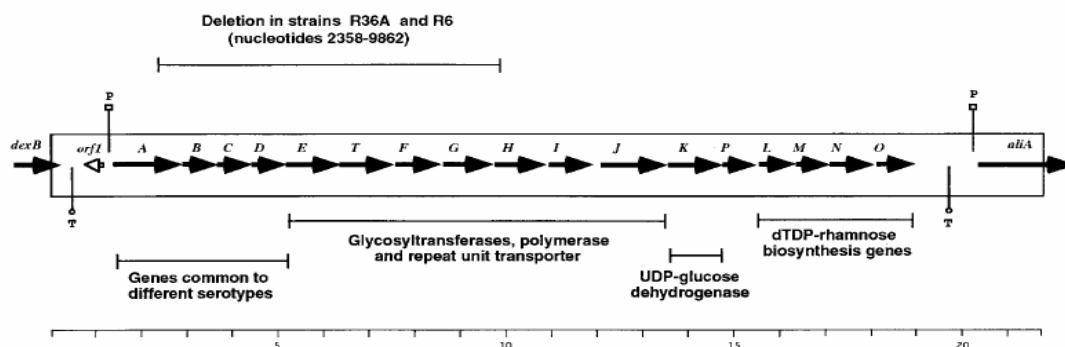
A. THE PNEUMOCOCCAL POLYSACCHARIDE CAPSULE IS THE MOST IMPORTANT VIRULENCE FACTOR.

The capsule polysaccharide forms a highly hydrated shell around the bacterium. During infection it inhibits phagocytosis by polymorphonuclear leukocytes [17, 77]. However, expression of the capsule reduces the ability of *S. pneumoniae* to adhere to respiratory epithelia and the transformation rate [78, 79]. The amount of capsule produced depends on phase variation, oxygen tension and close contact with an epithelial surface [80-82]. At least 90 different polysaccharide types have been identified [83]. Polysaccharides are distinguished by their chemical differences, and, in turn, the ability of the immune system to recognize these structural differences and to respond with specific antibodies against the antigen of each different type [84]. Each serotype has a structurally distinct capsular polysaccharide composed of repeating oligosaccharide units joined by glycosidic linkages. The capsular polysaccharide of some serotypes (2, 4, 6A, 6B, 7F, 8, 14, 19F, and 23F) seem to be covalently linked to the cell wall peptidoglycan [85].

B. THE GENE CLUSTERS INVOLVED IN CAPSULAR POLYSACCHARIDE SYNTHESIS SHOW A CONSERVED STRUCTURE.

Genes encoding for capsular polysaccharide biosynthesis are closely linked to each other in the pneumococcal chromosome forming a gene cluster located between *dexB* and *aliA* (Fig. 2) [86, 87]. This arrangement is found in all serotypes with the exceptions of serotypes 3 and 37. The first four genes are conserved between different serotypes and are involved in regulation and export of the CPS. They are followed by a central type-specific region, which encodes glycosyltransferases, a CPS polymerase and a CPS repeat unit flippase. The 3' region encodes enzymes for the biosynthesis of activated monosaccharide precursors, some of which are common to several CPS types, e.g. those encoding dTDP-L-rhamnose or UDP-glucuronic acid synthesis.

Figure 2. Genes involved in capsular production of type 2 *S. pneumoniae* [88]



Gene	Proposed function	Gene	Proposed function
A	Transcriptional modulator	I	N-acetyl-glycosamintransferase or lipopolysaccharide glycosyltransferase
B	Modulator of cap expression	J	Wzx homologue (transporter, "flippase")
C	Modulator of cap expression	K	UDP-glucose-6-dehydrogenase (Ugd)
D	Modulator of cap expression	P	UDP-galactopyranose mutase
E	Undecaprenyl-phosphate glucose-1-phosphate transferase	L	Glucose-1-phosphate thymidyltransferase (RmlA)
T	Rhamnosyltransferase	M	dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase (RmlC)
F	Galactosyltransferase or N-acetylglucosaminyltransferase	N	dTDP-D-glucose 4,6-dehydratase (RmlB)
G	Undecaprenyl-phosphate Galactosephosphotransferase	O	dTDP-6-deoxy-L-xylo-4-hexulose reductase (RmlD)
H	Wzy homologue (polymerase)		

C. THE BIOSYNTHESIS OF CPS FOLLOWS A BLOCKWISE PATHWAY: CPS

production requires a complex pathway, including synthesis of the activated monosaccharide nucleotide precursors, sequential transfer of each sugar to a lipid carrier to form biological repeating CPS units. These units are synthesized at the inner surface of the cytoplasmic membrane using undecaprenol pyrophosphate as the carrier before transportation across the cytoplasmic membrane by a flippase (encoded by *wzx*), subsequent polymerization by Wzy, and attachment to the cell surface (blockwise pathway) [89, 90]. So far, the biosynthetic pathways of serotypes 3 [91], 14 [92], and 19F [93] have been reported.

The current consensus is that CPS biosynthesis is governed exclusively by genes located in the cap locus, with the exceptions of serotype 3 and 37 [94-96] and with exceptions for genes encoding enzymes necessary for the synthesis of sugars with important housekeeping functions. As an example, glucose is generally transferred from UDP-glucose synthesized by a housekeeping gene *galU* located outside the capsule locus [87, 97, 98].

Table 2. Carbohydrates found in pneumococcal capsular polysaccharides [99]

Serotype	Carbohydrate
most frequently found carbohydrates in pneumococcal serotypes	α/β -D-glucose α/β -D-galactose α/β -L-rhamnose <i>N</i> -acetyl- α/β -D-glucosamine <i>N</i> -acetyl- α/β -D-galactosamine <i>N</i> -acetyl- α/β -D-mannosamine <i>N</i> -acetyl- α -L-fucosamine α/β -D-glucuronic acid
1	α -D-galacturonic acid 2-acetamido-4-amino-2,4,6-trideoxy- α -D-galactose
5	<i>N</i> -acetyl- α -L-pneumosamine 2-acetamido-2,6-dideoxy- α -D-xylo-hexos-4-ulose
7B, 19B, 19C	β -D-ribose
12A, 12F	<i>N</i> -acetyl- β -D-mannosaminuronic acid
19A	α -L-fucose

C.1. Biosynthesis and role of rhamnose: L-rhamnose is a deoxy sugar and synthesized by bacteria from glucose-1-phosphate (Glc-1-P). The immediate source of rhamnose in polysaccharides is dTDP-L-rhamnose. The rhamnose pathway is ubiquitous and highly conserved in both gram-positive and gram-negative bacteria. To date, neither rhamnose nor the genes required for its synthesis have been found in humans. Therefore, it has found recent interest as a therapeutic target [100].

The synthesis of dTDP-L-rhamnose requires the four *rml* genes (*rmlA*, *rmlC*, *rmlB*, *rmlD*). *Rml* genes can be found in the cap locus of the serotypes 2, 6A, 6B, 7A, 7B, 7C, 7F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24F, 27, 28A, 28F, 31, 32A, 32F, 40, 41A, 41F, 45, 48 (<http://www.sanger.ac.uk>). Serotype 1 cap does contain *rml* genes, but rhamnose is not part of the type 1 CPS [99]. If present the *rml* genes are always located at the 3' end of the capsule gene cluster and have the same gene order (*rmlA*, *rmlC*, *rmlB*,

rmlD). *Rml* genes of different serotypes appear to recombine at a higher frequency than that of housekeeping genes [97].

C.2. Biosynthesis and role of glucuronic acid: UDP-glucose is converted to UDP-glucuronic acid by action of the UDP-glucose dehydrogenase (Ugd) [101]. The following cap loci have a *ugd* gene: 1, 2, 3, 5, 8, 9A, 9L, 9N, 9V, 17A, 22A, 22F, 25F, 31, 38, 41A, 41F, and 45 (<http://www.sanger.ac.uk>). The capsule of serotypes 1 and 25F contain GalA and not GlcA [99].

D. PNEUMOCOCCUS CAN EXCHANGE CAPSULE GENES IN VIVO (CAPSULE SWITCH). Decades ago pioneering studies (that led to the discovery of DNA) showed that capsule phenotypes can be transferred between pneumococcal strains [102]. More recently it was shown that this involves the transfer of large DNA fragments, including the whole or part of the capsule operon (15 kb to >22 kb) [103]. Capsule switch is of epidemiological importance: the Spanish multi-resistant 23F strain has undergone several capsule switches to serotypes 3, 9N, 14, 19A, and 19F [103-105]. Among collections of clinical isolates the prevalence of capsule switch is approximately 1-2% [106].

E. EPIDEMIOLOGICAL AND ANIMAL STUDIES HAVE SHOWN CONSIDERABLE VARIATION BETWEEN PNEUMOCOCCAL SEROTYPES FOR THEIR TENDENCY TO COLONIZE THE HUMAN NASOPHARYNX OR CAUSE INVASIVE DISEASE [8, 107-111]. Typical “invasive” serotypes are 1, 4, 5, 7F, 9V and 14, and colonizing serotypes are 3, 6A, 6B, 18C, 19F and 23F. The underlying mechanism that renders some serotypes more invasive or colonizing than others is not understood. There is evidence from epidemiological and experimental studies, however, that the chromosomal background also plays a role in the virulence properties of specific pneumococcal clones [110, 112-114]. Some of the “invasive serotypes”, like 4, 9V and 14, are covered by PCV7, and others are not. The latter may become epidemiological more important. The inclusion of such emerging invasive serotypes into the conjugated vaccine would be necessary.

F. CAPSULE GENES MAY ALSO BE IMPORTANT FOR CENTRAL BACTERIAL METABOLISM. *S. pneumoniae* depends on external sugars for its energy requirement [115]. Simultaneous utilization of all available sugars would be

metabolically inefficient and would lead to slower growth. In *S. pneumoniae* glucose and sucrose are preferred sugars. However, the microorganism can also grow on many other sugars, when provided as the only carbon source, including maltose, cellobiose, raffinose and lactose. The ability to use preferred sugars depends on a regulatory process called carbon catabolite repression (CCR) [116, 117]. CCR causes silencing of genes specific for the utilization of nonpreferred sugars until the cell has exhausted the preferred sugar(s).

Most of the carbohydrates required for the polysaccharide synthesis are encoded by the capsule operon, but some precursors that occur in other cellular structures, such as glucose, N-acetylglucosamine of peptidoglycan, and ribitol of the teichoic acids, are catalyzed by enzymes involved in basic cellular metabolic functions and are encoded by genes outside the capsule operon [90]. According to the current opinion, the central metabolism does not use sugars synthesized by capsule enzymes [118]. However, previous studies have shown that deletion of genes involved in the production of polysaccharide capsule (*ugd* or *rml* genes) in *Cryptococcus neoformans* or *Streptococcus mutans* not only disrupted capsule production, but affected other cellular characteristics such as colony morphology, temperature sensitivity, growth, and sensitivity to the nutritional environment [119-121]. Also, there is evidence that in *C. albicans* glycosyltransferases (enzymes involved in polysaccharide biosynthesis) can have multiple cellular functions [122]. These findings suggest, that pneumococcal capsule genes may have multiple functions in the bacterial metabolism besides the biosynthesis of a polysaccharide capsule.

CHAPTER 2

The role of HexA for naturally occurring mutator phenotypes in *S. pneumoniae*

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The progress of this study has been delayed due to technical difficulties. Therefore, this chapter demonstrates the experiments which were performed so far and presents a strategy to overcome the technical problems.

SUMMARY

The spontaneous mutation frequency to rifampicin resistance of 50 clinical pneumococcal isolates revealed 28 strains with a normal mutation rate (5.1×10^{-8} , SD 2.2×10^{-8}) and 22 strains with an increased mutation rate (2.4×10^{-7} , SD 1.5×10^{-7}), designated mutators according to the definition by Morosini et al. [45]. Multilocus sequence typing (MLST) and pulsed-field gelelectrophoresis (PFGE) showed that 28 of the 50 isolates belonged to 7 clones and 22 were sporadic strains. Interestingly, the mutation rate to rifampicin resistance was conserved within clones. Clones of sequence type (ST)306, (ST)339 and (ST)344 exhibited a mutator phenotype. Clones of ST177, ST230, PFGE pattern 1 and PFGE pattern 2 had a normal mutation rate.

The role of HexA for the mutator phenotype observed in ST306 (serotype 1) and ST344 (non-typable) was investigated in vitro. First, a *hexA* deletion mutant of laboratory strain R6 (R6 Δ hexA) (with normal mutation rate) was generated. Multiple attempts to clone *hexA* into a plasmid for complementation studies failed. Finally, we were able to insert *hexA* directly into the chromosome of R6 *hexA* mutant. However, this strain harbors still remnants of its own *hexA*. In future experiments, the entire *hexA* gene including the promoter of a mutator strain will be transformed into R6 Δ hexA and the effect on the mutation rate will be investigated. Also, sequence analysis of the *hexA* gene in mutator clones ST306 and ST344 will be finished.

INTRODUCTION

Streptococcus pneumoniae is currently the most frequent cause of bacterial meningitis, community acquired bacterial pneumonia and acute otitis media. Genetic plasticity of pneumococci is the basis of new threats such as antibiotic resistance and vaccine escape mutants [123, 124]. *S. pneumoniae* is naturally transformable and horizontal gene transfer is the most frequent mechanism of genetic evolution in this pathogen. However, spontaneous mutation events also play a role. Especially, strains which exhibit a mutator phenotype possess the genetic flexibility to adapt rapidly to stressful conditions such as those experienced during antibiotic therapy [42-44]. In *S. pneumoniae* impairment of the mismatch repair systems MutY [47] and HexAB [45, 51, 58, 59] induce a mutator phenotype. Also, endogenous H₂O₂ production contributes to the spontaneous mutation rate [52].

Two *hex* genes, *hexA* and *hexB*, have been identified in *S. pneumoniae* [60]. Mutations in either of these genes abolishes mismatch repair and confers a mutator phenotype by elevating the mutation frequency 4- to 10-fold [45, 51, 58, 59]. HexA (a homologue to MutS in *E. coli*) recognizes the mismatched base pair, but the role of HexB has not been demonstrated in *S. pneumoniae*. By analogy with its *E. coli* homologue, it is likely to interact with HexA and other proteins in the mismatch repair complex and stimulate their activities, for example, by excision and replacement of the noncomplementary base [54, 61]. The Hex system repairs preferentially mismatches such as G/T, A/C, G/G and with lower efficiency C/T, A/A, T/T, A/G, C/C [48]. The Hex system acts both during transformation to correct donor strands and after DNA replication to remove misincorporated nucleotides. It is nick directed in *S. pneumoniae*, i.e. the daughter strand as well as the donor DNA during recombination are recognized by single-strand breaks [48].

Rifampicin resistance is caused by an alteration of the β subunit of RNA polymerase, the target of the antibiotic. Resistance is due to premature termination of DNA transcription. As previously shown in our laboratory [125], base changes mainly involved in rifampicin resistance are, for low-level resistance T \rightarrow A (I₅₄₅N) and A \rightarrow C (I₅₄₅L), and for high-level resistance C \rightarrow T (S₄₉₅F) and C \rightarrow G (H₄₉₉D). These base mutations are unlikely due to oxidative DNA damage. Mutations caused by oxidative damage to DNA result in G:C \rightarrow A:T transitions and G:C \rightarrow T:A transversions [53]. This is because guanine bases are particularly susceptible to oxygen radicals and oxidized guanine base (8-oxodG) introduces point mutations by pairing with adenine

or thymine, instead of cytosine [53, 54]. Hydrogen peroxide is shown to introduce exactly such oxidation-dependent mutations in *atpC* (encodes for H⁺ATPase) which leads to optochin resistance [52]. Deficiency in MutY induces preferentially G:C → T:A transversions [54], since it removes oxidized guanine base across from A in double-stranded DNA.

Therefore, this study investigated whether the Hex system, in particular HexA, is responsible for the mutator phenotype to rifampicin resistance observed in clinical *S. pneumoniae* strains. Fifty clinical pneumococcal isolates representing 7 clones (comprising 28 strains) and 22 sporadic strains were screened for a mutator phenotype defined as a mutation rate to rifampicin resistance of 10⁻⁷ or higher [45]. Twenty-eight strains showed a normal mutation rate (mean 5.1 × 10⁻⁸, SD 2.2 × 10⁻⁸). The remaining 22 strains exhibited a mutator phenotype (mean 2.4 × 10⁻⁷, SD 1.5 × 10⁻⁷). Interestingly, the mutation rate showed a clonal association. Three clones (ST306, ST339 and ST344) had a mutator phenotype and four clones (ST177, ST230, PFGE1 and PFGE2) exhibit a normal mutation rate.

Experiments attempting transformation of *hexA* from mutator clones into a non-mutator recipient were hampered by serious technical problems. However, lately a promising alternative strategy was found. Therefore, we hope, that it will be able to finish this study in the coming months.

MATERIALS AND METHODS

Bacterial strains

The clinical pneumococcal strains used in this study were obtained from a nationwide surveillance program collecting nasopharyngeal isolates since 1998 with short interruptions between January 2000 and March 2002. [Mühlemann unpublished data][9, 126]. Fifty strains were selected from this collection based on their serotypes and molecular typing by multilocus sequence typing (MLST) or pulsed-field gelelectrophoresis (PFGE). Strain R6 (spontaneous non-encapsulated derivative of D39), was kindly provided by Prof Philippe Moreillon (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland), pneumococcal strain R800 by Dr Jean-Pierre Claverys (Centre de Recherche de Biochimie et de Génétique Cellulaires du C.N.R.S, Toulouse, France) and *Escherichia coli* strain C600 by Prof Joachim Frey (Institute of Veterinary Bacteriology, Bern, Switzerland).

Bacterial culture

S. pneumoniae bacteria were grown on Columbia sheep blood agar (CSBA) plates at 37°C in a 5% CO₂-enriched atmosphere. Liquid culture was performed in brain heart infusion (BHI, pH 7.4-7.5) (Becton Dickinson and Company, le Pont de Claix, France) broth containing 5% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany) in a water bath at 37°C without shaking. For mutant or plasmid selection 2 µg/ml erythromycin (Sigma-Aldrich, Buchs, Switzerland) or 1 µg/ml tetracycline were added. Bacteria were stored at -80°C using Protect bacterial preservers (TSC, Heywood, U.K.). Optical density was measured at 600 nanometers (OD_{600nm}) using PerkinElmer™ Lambda-2 Spectrometer (PerkinElmer (Schweiz) AG, Schwerzenbach, Switzerland).

E. coli were grown in Luria-Bertani medium (Becton Dickinson and Company, le Pont de Claix, France) with or without 1.5% agar at 37°C with aeration. The antibiotic concentrations used for plasmid selection in *E. coli* were 500 µg/ml erythromycin and 15 µg/ml tetracycline.

Transformation of *E. coli* and *S. pneumoniae* was performed as described previously [125, 127].

Molecular typing

Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) were carried out as described previously [125, 128, 129].

Twenty two of the 50 clinical isolates showed a unique MLST type or PFGE pattern. The remaining 28 isolates fell into 7 clones. Two clones identified by PFGE (PFGE1 and PFGE2) were of serotype 6B and non-typable, respectively. The 5 other clones were characterised by MLST. All (n=3) isolates of sequence type (ST)306 were of serotype 1, isolates of ST344 (n=9) were non-typable. Isolates of ST177 (n=7), ST230 (n=2) and ST339 (n=2) were of serotype 19F.

Mutation rate to rifampicin-resistant phenotype

Five ml of BHI broth supplemented with 5% FCS was inoculated with one colony. When the bacteria reached an OD_{600nm} of 0.8, 200 µl of the culture were spread in duplicates on CSBA plates containing 0.5 µg/ml rifampicin. The plates were incubated for 48 hours prior to counting the colonies. In parallel, serial dilutions of the same culture were spread onto CSBA plates to determine the total viable cell count. Mutation rate was calculated as the number of rifampicin- or optochin-resistant colonies as a proportion of total viable cell count. Strains with a mutation frequency of 10⁻⁷ or higher were defined as mutators as described by Morosini et al. [45].

CLONING OF *hexA* GENE FROM A MUTATOR STRAIN INTO THE NON-MUTATOR PNEUMOCOCCAL RECIPIENT STRAIN R6

Aim

These experiments investigated whether *HexA* is responsible for the observed mutator phenotype in the Swiss clinical strain 110.58, which represents a non-encapsulated clone (Table 1, ST344) as previously described [126].

Overview of genetic manipulations performed

- 1) Inactivation of *hexA* gene of the pneumococcal recipient strain R6 (with normal mutation rate)
- 2) Construction of a plasmid containing *hexA*
 - a) Insertion of a linker into pMV158 using *E. coli* strains as recipients
 - successful cloning into *E. coli* strain C600, whereas pMV158 could not be transformed into *E. coli* strains Dh5 α and BL21
 - b) Amplification of *hexA* gene from R6 and mutator strain 110.58
 - c) Unsuccessful cloning of *hexA* into pMVL4 (pMV158 with linker) using *E. coli* strain C600, pneumococcal strains R800, R6 and R6 *hexA* mutant as recipient
 - d) Unsuccessful cloning of *hexA* into another restriction site of plasmid pMV158 (EcoRI of pLS1) or into an other plasmid (pJDC9)
- 3) Alternative strategy to 2): Successful insertion of *hexA* with its promoter into the chromosome of the *hexA*-deficient R6 strain.
- 4) Planned experiments:

A new mutant, lacking the whole *hexA* with its promoter, will be constructed to rule out possible recombination between the putative mutator *hexA* and the *hexA* remnants of the R6 *hexA* mutant.

After successful transfer of *hexA* from a mutator strain to the recipient strain R6, the mutation rate will be measured and compared with a) the recipient strain, and b) the recipient strain with inactivated chromosomal *hexA* complemented with its original *hexA*.

1) Inactivation of hexA gene of the recipient strain R6 (R6 Δ hexA)

A 2.6 kb fragment of *hexA* gene of strain 108.21 (non-encapsulated *S. pneumoniae*) was amplified (primers: hexA_f11: 5'-AGAGACAGAAAATGGCGATAGAAA-3', hexA_b2641: 5'-ATAGACAAAAGGGAGCGACAATG-3') and cloned into pGEM[®]-T Easy Vector (Promega, Wallisellen, Switzerland). A 1000 bp fragment of *hexA* was cut out by HindIII and was replaced with an erythromycin cassette (*ermB* of pJDC9). *S. pneumoniae* strain R6 was transformed with the whole plasmid and recombinants were selected on CSBA plates containing erythromycin (2 μ g/ml). Positive recombinants were analysed by Southern blot, PCR and phenotypically a 10-fold higher mutation rate to rifampicin compared to the wild-type was revealed (data not shown).

2) Construction of a plasmid containing hexA

a) The shuttle vector pMV158 [130], replicatable in *S. pneumoniae* and *E. coli*, was used. Transformation in our laboratory *E. coli* strains Dh5 α and BL21 was not possible. Finally, *E. coli* strain C600 turned out to be transformable with pMV158. Due to inappropriate restriction sites in plasmid pMV158, a linker (5'-CCAAGCTTGGGGGATCCCCCAAGCTTGGG-3') containing a BamHI restriction site (underlined) has to be inserted at the HindIII restriction site of pMV158 [131] (Fig. 1).

b) The *HexA* gene of strain R6 including the promoter region was amplified (primers: hexA_f2_BamHI: 5'-CGCGGATCCGCGGAGCTCCTTTTTGGACTTTTCTACT-3', hexA_b2641_BamHI: 5'-CGCGGATCCGCGATAGACAAAAGGGAGCGACAATG-3').

c) Plasmid pMV158 with the inserted linker (pMVL4) and the amplified *hexA* gene were digested and ligated. Successful ligation was confirmed with PCR using primers annealing to the plasmid pMVL4 and to the *hexA* (Fig. 2). Transformation, however, into *E. coli* strain C600 was not successful.

As the failure to transform the plasmids carrying the pneumococcal *hexA* gene might have resulted from a toxicity of the HexA product for *E. coli* cells [132], we tried to clone directly into *S. pneumoniae*. Direct cloning was performed in non-encapsulated pneumococcal strains R800, R6 and its *hexA* mutant, which exhibit a higher transformation efficiency [62, 79, 133]. In addition, the dephosphorylation of the plasmid prior to ligation was omitted, because *S. pneumoniae* takes up only single stranded DNA, which may lead to a recycling problem of the plasmid due to the

missing phosphate group [134]. However, also direct transformation in pneumococcus did not work.

d) To provide a new restriction site for cloning of *hexA* into pMV158, a 1000 bp fragment was cut out by EcoRI digestion and subsequently ligated, as described by Lacks et al. (pLS1 [135]). Unfortunately, cloning into this restriction site did also not work.

Chen et al. [136] presents an *E. coli* plasmid (pJDC9) constructed especially for cloning of pneumococcal DNA. It has been shown that strong pneumococcal promoter activity, interferes with maintenance of recombinant plasmid in *E. coli* [137]. Therefore, this vector contains a multiple cloning site surrounded by transcriptional terminators in order to protect from strong pneumococcal promoters. However, cloning of *hexA* into pJDC9 was not successful.

3) Insertion of *hexA* into the chromosome of R6 Δ *hexA*

A 4.2 kb fragment containing *hexA* with its promoter, flanked by 500 bp up- and downstream, was amplified from mutator strain 110.58 and transformed into R6 *hexA* mutant. Successful transformation should switch R6 *hexA* mutant from an erythromycin-resistant to an erythromycin-sensitive strain, due to the knock-out of the *ermB* cassette. Therefore, transformants were screened for erythromycin susceptibility by using replica plating. Insertion of *hexA* gene was confirmed by PCR.

Figure 1. Sequence spanning the HindIII restriction site of pMV158 and pMVL4.

Sequencing of pMV158 and pMVL4 showed that the linker (5'-CCAAGCTTGGGGGATCCCCCAAGCTTGGG-3') is inserted at the HindIII restriction site (underlined) in pMVL4.

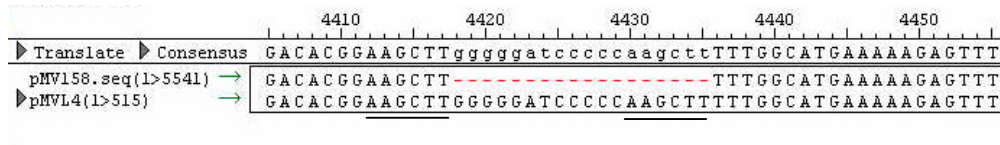
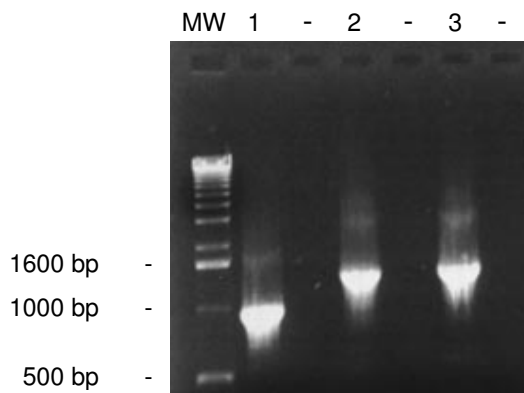
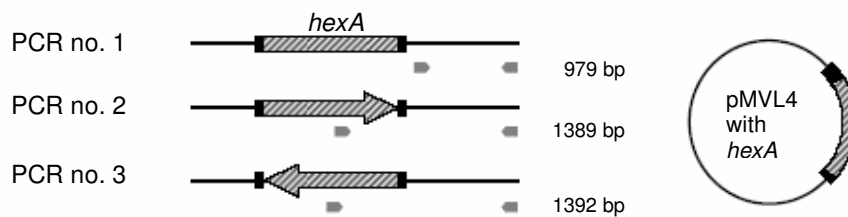


Figure 2. PCR to confirm the ligation of pMVL4 with hexA.

PCR using the ligation mix as template, with primers annealing to the plasmid pMVL4 and to the *hexA*, was performed. PCR no. 1: positive control. PCR no. 2 and 3 demonstrate that *hexA* is inserted in ON and OFF orientation.



MW: molecular weight marker

-: negative control, water instead of ligation mix as template

RESULTS

Spontaneous mutation rate to rifampicin resistance in clinical *S. pneumoniae* strains

Of the 50 isolates 22 were mutators and showed a mutation rate of 2.4×10^{-7} (SD 1.5×10^{-7}). Twenty-eight strains showed a normal mutation rate with a mean of 5.1×10^{-8} (SD 2.2×10^{-8}) (Table 1).

Association between clones and mutation rate

All strains of clones ST306, ST339 and ST344 were mutators with a mean mutation frequency of 2.6×10^{-7} (SD 0.6×10^{-7}). Clones PFGE1, PFGE2, ST177 and ST230 showed a mean mutation frequency of 8.3×10^{-8} (SD 2.9×10^{-8}) and comprise mainly non-mutator strains. ST230 is a pure non-mutator clone, whereas the other clones (PFGE1, PFGE2 and ST177) each have one mutator strain as well as non-mutator strains (Table 1). Clones ST306, ST339 and ST344 showed a significantly different mutation rate to rifampicin resistance compared with clones PFGE1, PFGE2 or ST177 ($p < 0.037$) (Fig. 3). One exception was that there was no significant difference between clone ST306 and PFGE1 ($p = 0.14$).

Table 1. Mutation rate to rifampicin resistance of 50 clinical pneumococcal isolates

	Strain	Serotype	Clone	Mutation rate to rifampicin resistance	
				Mutation rate	SD
Clinical non-mutator strains to rifampicin resistance (10 ⁻⁸ or lower)	101.51	6B	PFGE1	5.6 x 10 ⁻⁸	3.6 x 10 ⁻⁸
	104.39	19F	ST230	7.3 x 10 ⁻⁸	0
	105.38	19F	s	2.5 x 10 ⁻⁸	0
	105.67	nt	s	2.8 x 10 ⁻⁸	0
	106.27	6B	PFGE1	6.4 x 10 ⁻⁸	5.0 x 10 ⁻⁸
	106.68	19F	s	3.3 x 10 ⁻⁸	3.6 x 10 ⁻⁸
	107.24	19F	s	5.3 x 10 ⁻⁸	3.7 x 10 ⁻⁸
	107.81	19F	ST177	5.9 x 10 ⁻⁸	9.1 x 10 ⁻⁹
	108.10	23F	s	2.6 x 10 ⁻⁸	1.7 x 10 ⁻⁸
	108.21	19F	s	9.0 x 10 ⁻⁸	0
	108.34	19F	s	4.3 x 10 ⁻⁸	0
	108.46	nt	PFGE2	6.9 x 10 ⁻⁸	4.3 x 10 ⁻⁸
	108.76	19F	ST177	7.5 x 10 ⁻⁸	0
	109.24	19F	s	1.9 x 10 ⁻⁸	1.4 x 10 ⁻⁹
	109.71	19F	s	5.8 x 10 ⁻⁸	5.8 x 10 ⁻⁸
	110.02	19F	ST177	5.0 x 10 ⁻⁸	0
	110.13	7	s	4.1 x 10 ⁻⁸	0
	110.32	6	s	6.2 x 10 ⁻⁸	3.1 x 10 ⁻⁸
	110.36	19F	ST177	5.8 x 10 ⁻⁸	0
	110.68	6A	s	7.0 x 10 ⁻⁹	0
	111.06	19F	ST230	4.3 x 10 ⁻⁸	1.8 x 10 ⁻⁸
	111.46	19F	ST177	3.5 x 10 ⁻⁸	0
	111.72	19F	s	9.5 x 10 ⁻⁹	9.1 x 10 ⁻⁹
	201.17	17	s	8.7 x 10 ⁻⁸	1.7 x 10 ⁻⁸
201.41	19F	s	7.6 x 10 ⁻⁸	0	
202.10	19F	ST177	5.9 x 10 ⁻⁸	0	
204.26	23F	s	8.0 x 10 ⁻⁸	5.5 x 10 ⁻⁸	
204.50	23F	s	5.1 x 10 ⁻⁸	7.0 x 10 ⁻¹⁰	
			Mean	5.1 x 10⁻⁸	2.2 x 10⁻⁸
Clinical mutator strains to rifampicin resistance (10 ⁻⁷ or higher)	101.43	nt	ST344	8.0 x 10 ⁻⁷	0
	104.37	6B	PFGE1	2.4 x 10 ⁻⁷	2.7 x 10 ⁻⁷
	104.58	nt	ST344	3.0 x 10 ⁻⁷	1.3 x 10 ⁻⁷
	104.72	nt	ST344	4.1 x 10 ⁻⁷	1.2 x 10 ⁻⁷
	105.81	nt	PFGE2	1.1 x 10 ⁻⁷	2.1 x 10 ⁻⁸
	106.44	nt	ST344	1.6 x 10 ⁻⁷	0
	110.20	19F	ST177	1.1 x 10 ⁻⁷	8.2 x 10 ⁻⁸
	110.21	6B	s	1.2 x 10 ⁻⁷	6.3 x 10 ⁻⁸
	110.41	nt	ST344	1.0 x 10 ⁻⁷	0
	110.58	nt	ST344	2.5 x 10 ⁻⁷	1.0 x 10 ⁻⁷
	111.39	19F	ST339	3.7 x 10 ⁻⁷	0
	111.81	nt	ST344	3.0 x 10 ⁻⁷	1.4 x 10 ⁻⁷
	201.42	23F	s	1.2 x 10 ⁻⁷	5.5 x 10 ⁻⁸
	202.11	6B	s	1.1 x 10 ⁻⁷	3.8 x 10 ⁻⁸
	202.36	1	ST306	2.8 x 10 ⁻⁷	2.6 x 10 ⁻⁷
	202.66	nt	ST344	1.3 x 10 ⁻⁷	1.4 x 10 ⁻⁸
	202.80	19F	s	2.3 x 10 ⁻⁷	0
	203.15	nt	ST344	3.2 x 10 ⁻⁷	0
	203.53	19F	ST339	2.3 x 10 ⁻⁷	0
	204.68	6B	s	2.9 x 10 ⁻⁷	2.6 x 10 ⁻⁷
450	1	ST306	1.4 x 10 ⁻⁷	4.2 x 10 ⁻⁸	
777	1	ST306	1.6 x 10 ⁻⁷	5.6 x 10 ⁻⁸	
			Mean	2.4 x 10⁻⁷	1.5 x 10⁻⁷

nt, non-typable; s, individual pattern; SD, standard deviation.

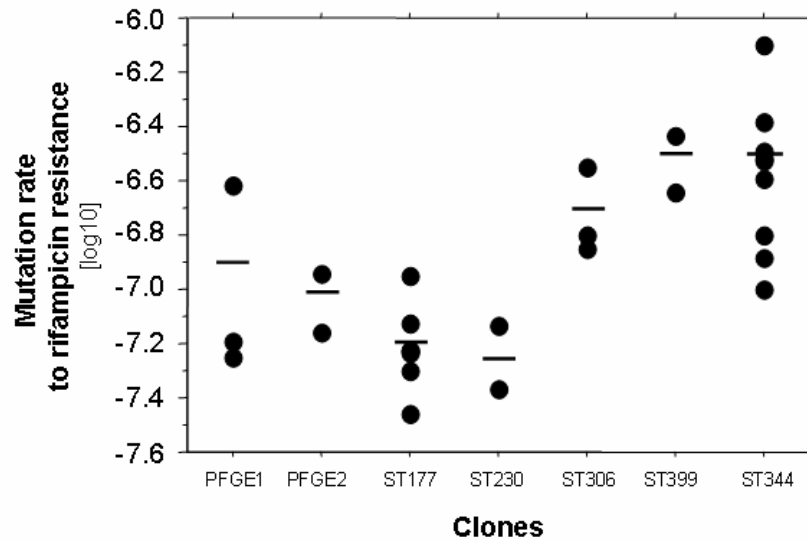


Figure 3. Mean mutation rate to rifampicin resistance of clones PFGE1, PFGE2, ST177, ST230, ST306, ST339 and ST344.

Mean values of the mutation rate to rifampicin resistance of each clone (dash) is shown. Mutation rate of each isolate (dots) represents mean values of duplicates from two independent experiments. All isolates of clones ST344 and PFGE2 were non-typable. Strains of ST306 were of serotype 1 and PFGE1 of serotype 6B. Clones ST177, ST230, ST339 harbor isolates from the serotype 19F.

DISCUSSION

This study first analyzed mutation rates to rifampicin resistance in a selection of 50 clinical, nasopharyngeal *S. pneumoniae* isolates, representing 7 clones and 22 sporadic strains. An elevated mutation rate (2.4×10^{-7}) was found in almost half of the isolates. This corresponds with the data of Morosini et al. [45]. They described a bimodal distribution of mutation frequencies with two peaks at 2.5×10^{-7} and 2.5×10^{-8} and showed that in *S. pneumoniae* mutators to rifampicin resistance exhibit a approximately 10-fold increased mutation rate.

An intriguing finding of our study was that mutation rate was a clonal characteristic and three (ST306, ST344, ST399) of the seven clones studied exhibited a mutator phenotype. ST306 and ST344 have been reported in the literature. Clone ST306 of serotype 1 belongs to one of three major lineages of serotype 1 found exclusively in Europe and North America [138]. ST306 was responsible for an epidemic increase of invasive *S. pneumoniae* infection associated with increased lethality in Sweden [30, 139]. The non-encapsulated clone ST344 has intrigued by its international spread [140], its association with bacterial conjunctivitis [140-142], its multidrug resistance [126, 140], and its peculiar capsule operon structure [126]. Whether the mutator phenotype has played a role for the successful spread of ST306 and ST344 remains open at this stage. Some of the features of ST344, such as multi-drug resistance and loss of the polysaccharide capsule by importation of genes in the capsule operon may be explained by a mutator phenotype especially if the underlying mechanism also mediates an increased transformation rate. Interestingly, some isolates of clone ST177 (serotype 19F) evolved to higher resistance levels to penicillin, probably by acquisition of *pbp2x* fragments from non-encapsulated isolates of ST344 [143].

We reasoned that deficiencies in the HexAB system might be the most likely mechanisms responsible for mutator phenotypes in our study strains. First, the relatively moderate increase in mutation rate corresponds with the observed mutations rate in Hex mutants (4- to maximum 30-fold increase, rarely greater than 10-fold) [45, 48, 51, 58, 59, 144, 145]. Second, HexA mutants also exhibit increased transformation rate [46, 51].

The main purpose of this study was, therefore, to investigate whether *hexA* is responsible for the mutator phenotype observed in clones ST306, ST399 and ST344. Sequence analysis of *hexA* for representative isolates of clones ST306 and ST344 is currently being performed in this laboratory. Morosini et al. compared the sequences

of *hexA* of 8 mutator and 5 non-mutator strains [45]. They found differences of a few amino acid changes (1 to 4 per strain) between mutators and non-mutators and concluded that these mutations could not be the cause of the mutator phenotype. However, the influence of these amino acid changes on structure or function of HexA have not been studied.

There is evidence that variations in *hexA* expression may influence the mutation rate. Humbert et al. [51] showed that a higher copy number of *hexA* enhances mismatch repair under saturating conditions. However, this likely does not apply for the mutators described in this study, since rifampicin resistance is based on a few point mutations in *rpoB* [125].

This study aimed at transforming the non-mutator strain R6 with *hexA* of a mutator strain. Numerous attempts to complement R6 Δ *hexA* with a plasmid containing a mutator *hexA* did failed. We first hypothesized that pneumococcal HexA might be toxic for *E. coli* cells used in the experiments. However, attempts to clone the plasmid with the ligated mutator *hexA* directly into *S. pneumoniae* also failed. At last, we successfully inserted *hexA* directly into the chromososome of R6 *hexA* mutant. However, recombination with the mutator *hexA* cannot be ruled out, since R6 Δ *hexA* was originally constructed for a complementation study and contained *hexA* remnants. Therefore, this experiments have to be repeated with a new R6 *hexA* mutant lacking the whole *hexA* including its promoter. If our results show that *hexA* is not responsible for the mutator phenotype, other genes like *hexB*, *mutT* or *mutY* will be investigated. Additional candidates are DNA polymerases. In *E. coli* it has been shown, that deficiency of the proofreading function of replicative DNA polymerases (DNA polymerase III) or overexpression of specialized DNA polymerases (Pol IV, Pol V and Pol II) lead to a mutator phenotype [42, 44, 54, 56, 57].

ACKNOWLEDGMENTS

This study was supported by a grant from the Swiss National Science Foundation to K.M., Grant No 3200-067998.

CHAPTER 3

Naturally occurring *S. pneumoniae* strain with increased mutation rate to optochin and hyperproduction of H₂O₂

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This chapter will be submitted for publication after the addition of the following experiments:

- a) influence of catalase supplementation on mutation rate of strains 110.68, 111.72 and D39
- b) hydrogen peroxide release of investigated strains and strain 111.72 at OD_{600nm} 0.15 in addition to the experiment performed at OD_{600nm} 0.3

SUMMARY

Determination of the mutation rate to optochin and rifampicin resistance of 32 clinical pneumococcal isolates, revealed two strains (strains 110.68 and 111.72) with an isolated increased mutation rate to optochin resistance (5.0×10^{-6} and 3.0×10^{-6} respectively) and a low mutation rate to rifampicin resistance (7.0×10^{-9} and 9.5×10^{-9} respectively). In the remaining 30 isolates, mutation rates to rifampicin and optochin showed a linear correlation ($p < 0.001$). This study investigated the role of H_2O_2 in inducing a mutator phenotype to optochin resistance only in the two strains 110.68 and 111.72. Hydrogen peroxide release was highest ($p < 0.032$) in strain 110.68 (strain 111.72 is currently under investigation), compared to seven clinical strains which exhibit a mutator phenotype to rifampicin, one clinical non-mutator strain and laboratory strains D39 and R6 (non-mutators). Expression of *spxB* gene in strain 110.68 was significantly upregulated during the early growth phase compared to reference strain D39.

Planned experiments: The mutation rate to optochin and rifampicin resistance of strains 110.68, 111.72 and D39 will be measured in the absence of H_2O_2 (catalase supplementation). We expect a significant reduction of the mutation rate to optochin resistance especially in strains 110.68 and 111.72, but almost no influence on the mutation rate to rifampicin resistance. Since, *spxB* expression peaked at OD_{600} 0.15, we will repeat the measurement of H_2O_2 production in all study strains at OD_{600} 0.15 (in addition to the experiment performed at OD_{600nm} 0.3).

INTRODUCTION

Streptococcus pneumoniae is the most frequent cause of bacterial meningitis, community acquired bacterial pneumonia and acute otitis media. Genetic plasticity of pneumococci add new threats such as antibiotic resistance and vaccine escape mutants [123, 124]. Horizontal gene transfer is the most frequent mechanism of genetic evolution in this naturally transformable pathogen [35]. However, spontaneous mutation events also play a role. Especially, strains which exhibit a mutator phenotype possess the genetic flexibility to adapt rapidly to stressful conditions such as those experienced during antibiotic therapy [42-44]. In *S. pneumoniae* impairment of the mismatch repair systems MutY [47] and HexAB [45, 51, 58, 59] induce a mutator phenotype. Also, endogenous H₂O₂ production contributes to the spontaneous mutation rate [52].

Pericone et al. [52] showed that high levels of H₂O₂ (>2 mM) increased the mutation frequency to optochin resistance up to 2.8×10^{-6} . Hydrogen peroxide causes oxidative DNA damage through hydroxyl radicals produced via the Fenton reaction [65, 66]: $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^\circ + \text{OH}^-$. Guanine bases are particularly susceptible to oxygen radicals. Oxidized guanine base (8-oxodG) introduces point mutations by pairing with adenine or thymine, instead of cytosine [54]. The Mut system, also found in *S. pneumoniae* [47], acts against these oxidation-induced mutations. MutY removes oxidized guanine base across from A in double-stranded DNA and prevents therefore G:C → T:A transversions [54, 67-71]. Finally, H₂O₂ induced mutations result in G:C → A:T transitions and less frequently in G:C → T:A transversions (repaired by MutY) [53].

Pericone et al. [52] sequenced the H⁺ATPase gene, which encodes for optochin susceptibility or resistance, and found the expected hierarchy of single-base-pair changes (G → A > G → T) in the optochin-resistant strains. In addition, they demonstrated that the mutation frequency to optochin resistance was 10- to 200-fold lower in the absence of detectable levels of H₂O₂. Resistance to rifampicin has been shown to be linked to amino acid changes in Clusters I to III of *rpoB* (β subunit of RNA polymerase) [125, 146, 147]. Amino acid changes mediating low-level resistance are mainly due to T → A and A → C base pair changes and for high-level resistance C → T and C → G mutations. Therefore, point mutations in *rpoB* mediating rifampicin resistance, have not the mutational spectra of oxidation-dependent DNA damage.

We hypothesized that naturally occurring mutator phenotypes induced by hyperproduction of H₂O₂ could exist and that such strains should have a high mutation rate to optochin resistance, but normal rate to rifampicin resistance. Screening of 32 selected, clinical, nasopharyngeal isolates for mutation rate to optochin and rifampicin resistance identified two strains (110.68 and 111.72) with an increased mutation rate to optochin (5.0×10^{-6} and 3.0×10^{-6}) and a normal mutation rate to rifampicin (7.0×10^{-9} and 9.5×10^{-9}). In the remaining 30 isolates mutation rate to optochin and rifampicin resistance, respectively, showed a significant, linear relationship ($p < 0.001$). Strain 110.68 had a significantly increased production of H₂O₂ and increased levels of *spxB* expression, which peaked in the early growth phase. Currently, we are investigating the mutation rate to optochin and rifampicin resistance of strains 110.68, 111.72 and D39 in the absence of H₂O₂ (catalase supplementation) and we will repeat the measurement of H₂O₂ production in all study strains at OD₆₀₀ 0.15, since *spxB* expression peaked at OD₆₀₀ 0.15.

MATERIALS AND METHODS

Bacterial strains

The clinical pneumococcal strains used in this study were obtained from a nationwide surveillance program collecting nasopharyngeal isolates since 1998 with short interruptions between January 2000 and March 2002 [Mühlemann unpublished date][9, 126]. As described in chapter 2 of this thesis, 50 isolates were selected for determination of mutation rate to rifampicin resistance. In 32 of these 50 isolates mutation rate to optochin resistance was also determined (as described below).

Pneumococcal laboratory strain D39 (serotype 2) [133], was kindly provided by Prof Jeffrey Weiser (University of Pennsylvania, Philadelphia, U.S.A) and strain R6 (spontaneous non-encapsulated derivative of D39) by Prof Philippe Moreillon (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland).

The minimal inhibitory concentrations (MICs) of azithromycin, chloramphenicol, ciprofloxacin, erythromycin, gatifloxacin, levofloxacin, ciprofloxacin, penicillin, tetracycline, trimethoprim-sulfamethoxazole (TMP/SMX), and vancomycin were determined by the E-test method (AB Biodisk, DMD AG, Arlesheim, Switzerland) according to the methods recommended by the National Committee for Clinical Laboratory Standards (NCCLS) or isolates were tested against erythromycin, penicillin, TMP/SMX and ofloxacin by the disc diffusion method as previously described [9].

Bacterial culture

Bacteria were grown on Columbia sheep blood agar (CSBA) plates at 37°C in a 5% CO₂-enriched atmosphere. Liquid culture was performed in brain heart infusion (BHI, pH 7.4-7.5) (Becton Dickinson and Company, le Pont de Claix, France) broth containing 5% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany) in a water bath at 37°C without shaking. If stated 80 U/ml catalase (Sigma-Aldrich, Buchs, Switzerland) was added to BHI with 5% FCS. Bacteria were stored at -80°C using Protect bacterial preservers (TSC, Heywood, U.K.). Optical density was measured at 600 nanometers (OD_{600nm}) using PerkinElmer™ Lambda-2 Spectrometer (PerkinElmer (Schweiz) AG, Schwerzenbach, Switzerland).

Mutation rate to rifampicin and optochin resistance

Five ml of BHI broth supplemented with 5% FCS was inoculated with one colony. When the bacteria reached an OD_{600nm} of 0.8, 200 µl of the culture were spread in duplicates on CSBA plates containing 0.5 µg/ml rifampicin or 5 µg/ml optochin. The plates were incubated for 48 hours prior to counting of the colonies. In parallel, serial dilutions of the same culture were spread onto CSBA plates to determine the total viable cell count. Mutation rate was calculated as the number of streptomycin- or optochin-resistant colonies as a proportion of total viable cell count. Strains with mutation rates of 10⁻⁷ or higher to rifampicin resistance were defined as mutators as described by Morosini et al. [45]. No cut-off was defined for the mutation rate to optochin resistance.

Hydrogen peroxide release assay

The release of H₂O₂ by *S. pneumoniae* was determined by a modification of the protocol provided in the Amplex[®] Red hydrogen peroxide/peroxidase assay kit instruction manual (Molecular Probes, Eugene, Oregon, U.S.A).

Overnight culture was 1:100 diluted, incubated at 37°C and a sample of 2.5 ml were withdrawn at OD_{600nm} 0.3. Sample was pelleted at 5000 rpm, 4°C for 10 minutes and washed with Hanks medium (4°C) (Gibco[™] Hanks' Balanced Salt Solution (no phenol red), Invitrogen, Carlsbad, California, U.S.A). After an additional centrifugation the pellet was dissolved and 20-fold diluted in BHI with 5% FCS. 50 µl Hanks medium containing 0.2 U/ml horseradish peroxidase and 100 µM Amplex Red reagent were added to 50 µl bacterial culture. Standard curves were generated with fresh dilutions of 30% H₂O₂. The reactions were incubated at 37°C, and the absorbance was read at 563 nm (SpectraMax GeminiXS, Molecular Devices, Sunnyvale, California, U.S.A) every minute for 15 minutes. In parallel, serial dilutions of the same bacterial culture were spread onto CSBA plates to determine the total cell count. The H₂O₂ release of investigated strains is presented as a ratio compared with the H₂O₂ release of strain D39 or R6 for non-encapsulated strains, measured at the same day. For each experiment three independent measurements were performed on different days.

RNA isolation for RT-PCR

Bacteria were grown in BHI with 5% FCS to an OD_{600nm} of 0.05, 0.15, 0.25, 0.35, 0.45, 0.55 or 0.65, twice the culture volume of RNAprotect™ (Qiagen AG, Hombrechtikon, Switzerland) was added to prevent further transcription and to prevent RNA degradation. After vortexing and a 5 min incubation at room temperature (RT), the bacteria were pelleted by centrifugation for 10 min at 5000 g. The pellet was resuspended in 200 µl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH=8) containing 600 µg lysozyme (Roche Diagnostics GmbH, Mannheim, Germany). After 10 min at RT with repeated vortexing, 700 µl RLT buffer (Qiagen RNeasy® Mini kit), containing β2-mercaptoethanol (Merck KGaA, Darmstadt, Germany), was added and the tubes vortexed. The mixture was transferred to a 1.5 ml tube containing 0.05 g 100 µm acid-washed glass beads (Sigma-Aldrich, Buchs, Switzerland) and vibrated for 10 min at half maximum speed using a Mickle Vibratory Tissue Disintegrator (Mickle Laboratory Engineering, Gomshall, U.K.). The mixture was then centrifuged and RNA extracted from the supernatant using Qiagen RNeasy® Mini kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's instructions. The RNA recovered was treated with DnaseI (Stratagene Europe, Amsterdam, Netherlands) according to the manufacturer's instructions to remove any contaminating DNA. RNA concentration and purity were determined by measuring absorbance at both 260 nm and 280 nm (Lambda-2 Spectrometer, PerkinElmer (Schweiz), Schwerzenbach, Switzerland).

Quantitative gene expression using a cRNA standard curve

Quantitation of absolute mRNA copy numbers by real-time RT-PCR was performed by using a standard curve generated based on *in vitro* transcribed RNA (cRNA) as previously described [148, 149].

The primer pair for the standard curve was designed to flank the real-time PCR target sequence of *spxB* gene (spxB_F1_T7: 5'-TAATACGACTCACTATAGGGAGAGTGGAATAGTAAAAATTTGGAGAACG-3', spxB_Bac1: 5'-CGATCTTTTAAAGTTCTGCTCTATG-3'). The forward primer contained the T7 RNA polymerase promoter (T7 promoter is underlined) sequence at the 5' end to allow *in vitro* transcription from PCR product using the MEGAscript T7 High Yield Transcription Kit (Ambion, Cambridgeshire, U.K.) followed by treatment with DNase I (Stratagene Europe, Amsterdam, Netherlands) according to

manufacturer's protocol. Standard curve cRNA was purified using the RNeasy Kit (Qiagen AG, Hombrechtikon, Switzerland) according to the RNA clean-up protocol supplied by the manufacturer. Size and integrity of transcripts was verified on a 1.2% formaldehyde gel. cRNA was quantified by spectrophotometry at 260 nm (Lambda-2 Spectrometer, PerkinElmer (Schweiz), Schwerzenbach, Switzerland). Concentration of cRNA was converted into copy number according to the following equation: N (copies/ μ l) = C (cRNA in μ g/ μ l) / K (fragment size in bp) * 182.5×10^{12} (Avogadro constant). 3 μ l standard curve cRNA of serial ten-fold dilutions (10^{12} to 10^0 copies/ μ l) and 3 μ l RNA extracted from 10 ml bacterial culture of OD_{600nm} 0.05, 0.15, 0.25, 0.35, 0.45, 0.55 and 0.65 was reversed transcribed to cDNA using Superscript II (Amersham, Buckinghamshire, U.K.) and random hexamer primers (Promega, Wallisellen, Switzerland) according to supplier's protocol. For the cultures of each OD_{600nm} from which we extracted RNA, serial dilutions were spread onto CSBA plates to determine the cell count (cfu). Quantification of gene expression was achieved by real time RT-PCR using TaqMan primers and probes created by the Assay-by-Design™ Service of Applied Biosystems (Rotkreuz, Switzerland) based on the most conserved regions of the *spxB* gene in *S. pneumoniae* strain TIGR4 (AE005672), strain R6 (AE008442), strain D39 (AY254852) and strain AB15 (AY254854). (forward primer *spxB*-tgt3F: 5'- ACAGGTTCTGCTTACCGTGTTG-3', reverse primer *spxB*-tgt3R: 5'- AGGAAAAGAACTGTGTCTGCTTCAA-3' and probe *spxB*-tgt3M2: 6-FAM- TCGTTGGCTGGTTTCCAA-MGB). cDNA was diluted 4-fold in the assay and an RT-negative control was performed for every sample. Real-time RT-PCR was performed in 96-well plates using ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). The experiment was performed on three different days and real-time RT-PCR was carried out in triplicates. Finally, for the standard curve, copy number was plotted against cycle-of-threshold (Ct) value (Applied Biosystems, ABI PRISM SDS 7000 Software v1.1) and the Ct values and the cell count of the extracted RNA cultures were used to calculate the copy number of the samples per 10^2 cfu.

Statistical analyses

Statistical analyses were done in StatView® version 5.0 (SAS Institute Inc., Cary, NC). Proportions were compared with the Chi square test or Fisher's exact test as appropriate. Differences between means were assessed by the Student's t-test. A cut-off of $p \leq 0.05$, two tailed, was used for all statistical analyses.

RESULTS

Mutation rate to optochin and rifampicin resistance

The mutation rate to optochin and rifampicin resistance were determined of 32 clinical isolates. Non-mutators had a mean mutation frequency to rifampicin of 4.7×10^{-8} (SD 2.3×10^{-8}) and a mean rate to optochin resistance of 8.9×10^{-7} (SD 1.4×10^{-6}), respectively (Table 1). Mutator strains exhibited a 5- to 10-fold higher mutation rate to rifampicin (mean 2.5×10^{-7} , SD 1.6×10^{-7}) and a mutation frequency of 9.3×10^{-7} (SD 1.0×10^{-6}) to optochin resistance.

Two strains were of special interest (strains 110.68 and 111.72). They showed a high mutation rate to optochin resistance (10^{-6}), but a normal mutation rate to rifampicin resistance (10^{-9}). This 1000-fold difference in the mutation frequency to optochin and rifampicin resistance was unique among the isolates studied. After, exclusion of these two strains, regression analysis showed a significant linear association between the mutation rate to optochin and rifampicin resistance ($p < 0.0001$) (Fig. 1A). Strains 110.68 and 111.72, however, exhibit the lowest measured rifampicin resistance (7.0×10^{-9} and 9.5×10^{-9} , respectively) and their optochin resistance belongs to highest observed mutation rates (5.0×10^{-6} and 3.0×10^{-6}) (Fig. 1B).

Table 1. Mutation rates to rifampicin and optochin resistance

	Strain	Serotype	Mutation rate to rifampicin resistance		Mutation rate to optochin resistance	
			Mutation rate	SD	Mutation rate	SD
Clinical non-mutator strains to rifampicin (10^{-8} or lower) and optochin resistance	101.51	6B	5.6×10^{-8}	3.6×10^{-8}	9.3×10^{-7}	1.0×10^{-6}
	104.39	19F	7.3×10^{-8}	0	2.0×10^{-7}	1.2×10^{-7}
	105.38	19F	2.5×10^{-8}	0	2.4×10^{-7}	1.4×10^{-7}
	108.46	Nt	6.9×10^{-8}	4.3×10^{-8}	2.1×10^{-7}	1.1×10^{-7}
	108.76	19F	7.5×10^{-8}	0	2.9×10^{-7}	1.8×10^{-7}
	109.24	19F	1.9×10^{-8}	1.4×10^{-9}	1.7×10^{-6}	2.7×10^{-6}
	109.71	19F	5.8×10^{-8}	5.8×10^{-8}	1.2×10^{-7}	0
	110.32	6	6.2×10^{-8}	3.1×10^{-8}	1.2×10^{-7}	3.6×10^{-8}
	110.36	19F	5.8×10^{-8}	0	2.8×10^{-7}	2.9×10^{-7}
	110.68	6A	7.0×10^{-9}	0	5.0×10^{-6}	5.2×10^{-6}
	111.06	19F	4.3×10^{-8}	1.8×10^{-8}	1.2×10^{-7}	4.1×10^{-8}
	111.46	19F	3.5×10^{-8}	0	2.0×10^{-7}	6.7×10^{-8}
	111.72	19F	9.5×10^{-9}	9.1×10^{-9}	3.0×10^{-6}	5.9×10^{-6}
	204.26	23F	8.0×10^{-8}	5.5×10^{-8}	1.3×10^{-7}	3.2×10^{-8}
	Mean		4.7×10^{-8}	2.3×10^{-8}	8.9×10^{-7}	1.4×10^{-6}
Clinical mutator strains to rifampicin (10^{-7} or higher) and optochin resistance	101.43	nt	8.0×10^{-7}	8.7×10^{-7}	1.4×10^{-6}	8.7×10^{-7}
	104.37	6B	2.4×10^{-7}	2.7×10^{-7}	1.5×10^{-6}	1.8×10^{-6}
	104.58	nt	3.0×10^{-7}	1.3×10^{-7}	4.8×10^{-7}	1.9×10^{-7}
	104.72	nt	4.1×10^{-7}	1.2×10^{-7}	4.5×10^{-7}	2.3×10^{-7}
	105.81	nt	1.1×10^{-7}	2.1×10^{-8}	1.6×10^{-7}	9.5×10^{-8}
	106.44	nt	1.6×10^{-7}	0	3.4×10^{-7}	2.5×10^{-7}
	110.21	6B	1.2×10^{-7}	6.3×10^{-8}	1.5×10^{-7}	8.7×10^{-8}
	110.41	nt	1.0×10^{-7}	0	1.3×10^{-7}	6.0×10^{-8}
	110.58	nt	2.5×10^{-7}	1.0×10^{-7}	1.8×10^{-7}	5.2×10^{-8}
	111.39	19F	3.7×10^{-7}	0	3.7×10^{-7}	4.5×10^{-7}
	111.81	nt	3.0×10^{-7}	1.4×10^{-7}	2.4×10^{-6}	1.2×10^{-6}
	202.11	6B	1.1×10^{-7}	3.8×10^{-8}	1.0×10^{-7}	2.3×10^{-8}
	202.36	1	2.8×10^{-7}	2.6×10^{-7}	8.7×10^{-7}	6.1×10^{-7}
	202.66	nt	1.3×10^{-7}	1.4×10^{-8}	2.6×10^{-6}	5.1×10^{-7}
	202.80	19F	2.3×10^{-7}	0	4.9×10^{-7}	8.0×10^{-7}
	203.15	nt	3.2×10^{-7}	0	9.7×10^{-7}	1.0×10^{-6}
	450	1	1.4×10^{-7}	4.2×10^{-8}	7.2×10^{-7}	3.9×10^{-7}
777	1	1.6×10^{-7}	5.6×10^{-8}	3.6×10^{-6}	3.8×10^{-6}	
	Mean		2.5×10^{-7}	1.6×10^{-7}	9.3×10^{-7}	1.0×10^{-6}

nt, non-typable; SD, standard deviation.

Fig. 1A

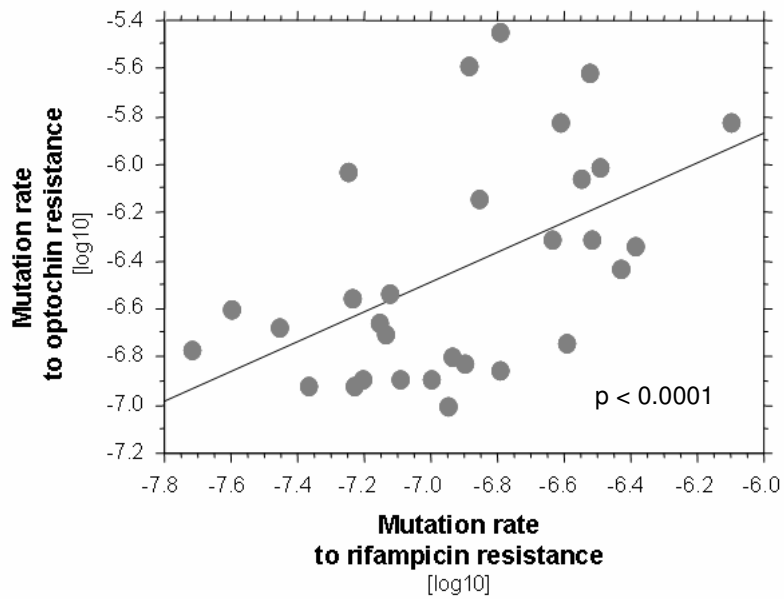


Fig. 1B

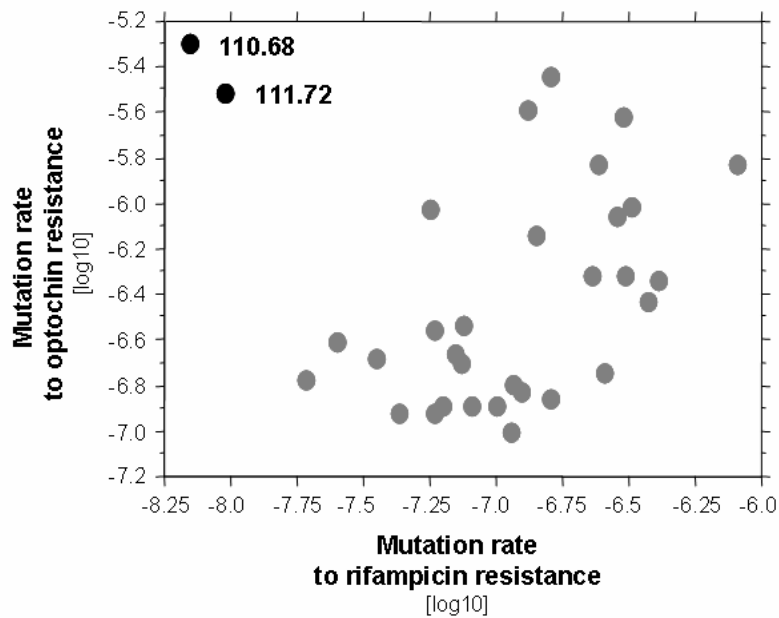


Figure 1. Mutation rates of 32 clinical pneumococcal isolates to optochin and rifampicin resistance.

A) Linear regression analysis of the association between the mutation rate to rifampicin and optochin resistance including 30 of 32 isolates. Strains 110.68 and 111.72 were excluded. **B)** Mutation rate to rifampicin resistance plotted against

optochin resistance of all 32 clinical isolates (including strains 110.68 and 111.72). Presented are mean values of each isolate (dots) of 2 to 4 independent experiments.

Hydrogen peroxide release

This experiment investigated differences in H₂O₂ release between strain 110.68 with an increased mutation rate to optochin (10⁻⁶) only and strains with normal mutation rates (n=2) or strains with a mutator phenotype to rifampicin resistance (n=7). Hydrogen peroxide release was significantly higher in strain 110.68 compared to the other strains (p<0.032 for all strains except strain 202.36) (Fig. 2).

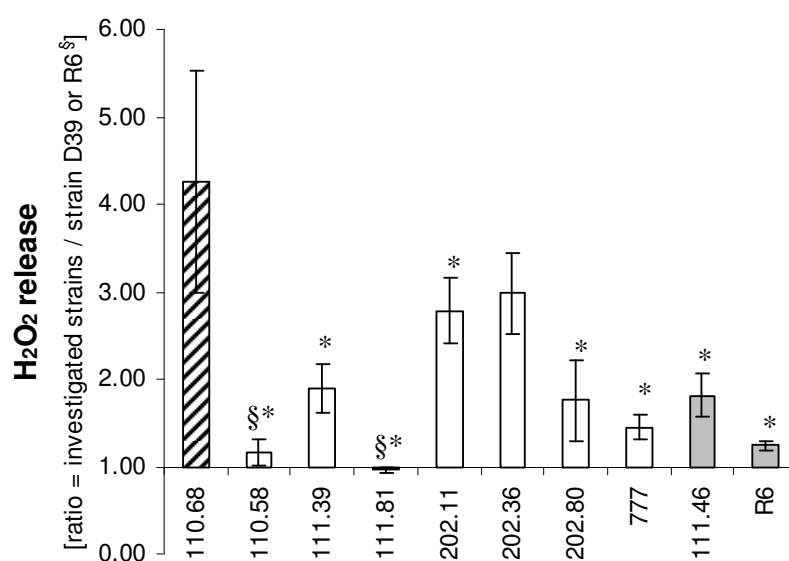


Figure 2. Hydrogen peroxide release of pneumococcal strains at OD₆₀₀ 0.3.

Hydrogen peroxide release is displayed as the ratio of H₂O₂ release for the investigated strain compared to strain D39 for encapsulated strains and R6 for the non-encapsulated strains 110.58 and 111.81 (measured at the same day). Presented are mean values of duplicates from three independent experiments (± SE). Striped bar indicates the strain 110.68 with increased mutation rate to optochin resistance only, open bars indicate strains with a mutator phenotype to rifampicin resistance, and grey bars stand for strains with normal mutation rate to rifampicin resistance. * p<0.05; the p-values were calculated by comparing the H₂O₂ release (ratio) of strain 110.68 with each individual strain.

***SpxB* gene expression of strain 110.68 compared to strain D39**

SpxB transcription was investigated in strain 110.68 and in strain D39. In both strains *spxB* transcription peaked at OD_{600nm} 0.15 and decreased thereafter (Fig. 3). Strain 110.68 showed a significantly higher *spxB* transcription during the early growth phase (OD_{600nm} 0.05 and 0.15). The difference in *spxB* transcription between strain 110.68 and D39 was greatest (6-fold) at OD₆₀₀ 0.05 (p=0.002).

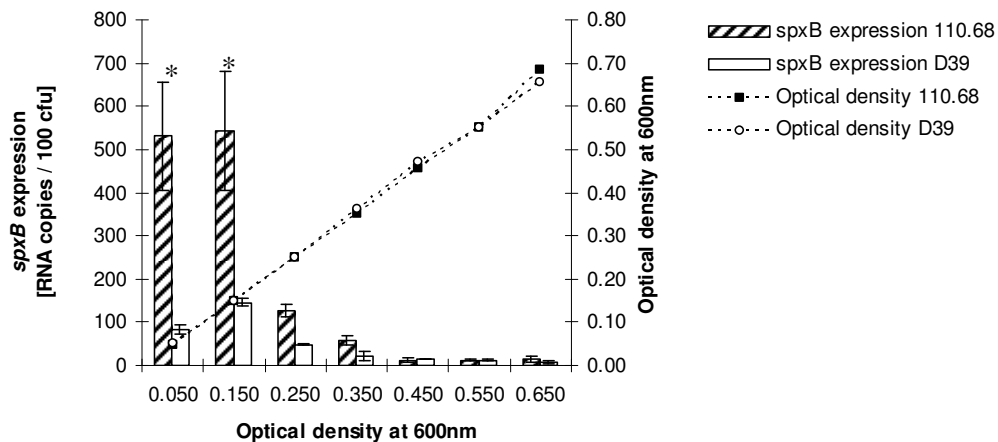


Figure 3. *SpxB* gene expression of strains 110.68 and D39.

Left axis: The *spxB* transcription levels were determined by real-time RT-PCR expressed as the copy number per 100 cfu. Presented are mean values of triplicates from 3 independent experiments (\pm SE). * p<0.05; the p-values were calculated by comparing the *spxB* expression of strain 110.68 with the *spxB* expression of strain D39 at each optical density. **Right axis:** Presented are the mean values of the optical densities at 600nm obtained during the 3 independent experiments.

Antibiotic resistance pattern of strains 110.68 and 111.72

Strain 110.68 is sensitive to chloramphenicol (MIC 2.0 μ g/ml), erythromycin (MIC 0.064 μ g/ml), levofloxacin (MIC 0.75 μ g/ml), penicillin (MIC 0.023 μ g/ml), tetracycline (MIC 0.094 μ g/ml) and vancomycin (MIC 0.5 μ g/ml) and resistant to TMP/SMX (MIC 1.0 μ g/ml). The MIC of ciprofloxacin was 0.75 μ g/ml (breakpoints for ciprofloxacin has not been established).

Strain 111.72 is susceptible (disc test) to erythromycin, penicillin, TMP/SMX, levofloxacin.

DISCUSSION

This study investigated, whether increased H₂O₂ production is a naturally occurring mechanism of mutator phenotypes. Based on previous work by the group of Weiser et al. [52], high mutation rate to optochin resistance combined with a normal mutation rate to rifampicin resistance were used as indicators for hyperproduction of H₂O₂. Among a selection of 32 clinical nasopharyngeal isolates, two candidate strains with increased mutation rate to optochin resistance only were identified and one of them, strain 110.68, was further investigated (the other is still under investigation).

Hydrogen peroxide release was significantly higher in strain 110.68 than strains with normal or increased mutation rate to rifampicin. This supports our hypothesis, that mutator phenotypes due to H₂O₂ hyperproduction do occur and can be identified on the basis of a high mutation rate to optochin resistance and normal mutation rate to rifampicin resistance.

Expression of *spxB* gene was found to be significantly upregulated in strain 110.68 compared to strain D39 in the early growth phase. This indicates that at least in strain 110.68 (hyper-)production of H₂O₂ is controlled at the transcriptional level. However the mechanism leading to higher *spxB* expression levels remains unknown. Peak expression of *spxB* during the early growth phase corresponds to the observation of Lee et al. [150], who showed an induction of pyruvate oxidase (SpxB) in the early growth phase using 2-D gel electrophoresis. The difference between strains 110.68 and D39 in *spxB* expression was greatest at OD₆₀₀ 0.05 and 0.15. We could expect that the differences in H₂O₂ production should also be greatest at these ODs and already diminished at OD₆₀₀ 0.3, at which measurements were performed. Therefore, we will repeat the H₂O₂ measurement at an OD₆₀₀ 0.15. Higher H₂O₂ production during the early growth phase coincides with the time of natural competence in *S. pneumoniae*. We suggest that the bacteria use the toxic effect of H₂O₂ on DNA to generate mutated DNA fragments which can be taken-up during competence and offer the possibility for evolution if needed. This may involve DNA released from a subfraction of pneumococcal cells during competence [36, 38, 39] or foreign DNA. Also, H₂O₂ has been shown to inhibit other bacterial species present in the nasopharynx [63]. *S. pneumoniae* may need this competitive factor above all in the early growth phase, when establishing colonization. Whether H₂O₂ also contributes to DNA release is further investigated in chapter 4.

Jorgensen et al. [151] showed that amino acid changes mainly due to G to A substitution in *parC* (topoisomerase IV) led to resistance to quinolones. This indicates that mutations in *parC* result from oxidative stress. The increased H₂O₂ production of strain 110.68 may be an advantage in acquiring quinolone resistance. However, ciprofloxacin MIC measured for strain 110.68 was not indicative of any mutations.

In conclusion, we showed that the increased mutation rate to optochin resistance in strain 110.68 is based on overexpression of *spxB* and in consequence on hyperproduction of H₂O₂. This mechanism is likely to be different from the one(s) responsible for the mutator phenotype in the remaining strains. Although, the latter represent the majority of mutator strains, one (3.1% possibly two 6.2%) of 32 mutator strains were due to H₂O₂ hyperproduction.

To validate further our observation, the mutation rate to optochin and rifampicin resistance of strains 110.68, 111.72 and D39 will be measured in the absence of H₂O₂ (catalase supplementation). We expect a significant reduction of the mutation rate to optochin in H₂O₂ hyperproducers, but almost no influence on the mutation rate to rifampicin resistance in all three strains.

ACKNOWLEDGMENTS

This study was supported by a grant from the Swiss National Science Foundation to K.M., Grant No 3200-067998. We thank Suzanne Aebi for her tremendous effort and laboratory assistance.

CHAPTER 4

Role of pneumococcal *spxB* gene in DNA release

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This chapter will be submitted for publication after the addition of experiments relating to the *recA* and *comC* expression in D39 Sm^R and its *spxB* mutant.

SUMMARY

In *S. pneumoniae* lysis of a subfraction of bacterial cells occurs during competence. The lytic process has been shown to be mediated by the bacteriocin system CibABC and the cell-wall hydrolases CbpD, LytA, and LytC. However, the release of some of the DNA seems not to be affected by competence. This study investigated the role of *spxB* and endogenously produced H_2O_2 in pneumococcal cell lysis using the laboratory strain D39. It was found, that *spxB* expression and endogenous H_2O_2 production peaked during the early growth phase which coincides with competence. DNA release was slightly reduced in the presence of catalase during the early growth phase. However, isogenic *spxB* knock-out mutants showed significantly reduced DNA release and transformation rate. Supplementation with H_2O_2 with or without the addition of acetate (restores the level of acetyl phosphate, another end product of SpxB) did not influence DNA release in the mutant.

In conclusion, the pneumococcal *spxB* gene plays a role for the competence associated DNA release from a subfraction of bacterial cells, which is not mediated by a toxic effect of H_2O_2 . Also, neither of the two products of SpxB (H_2O_2 and acetyl phosphate) seem to act as messengers for DNA release. Whether there is a link between *spxB* and competence is currently investigated in gene expression studies of the competence genes *recA* and *comC*.

INTRODUCTION

S. pneumoniae is among at least 40 bacterial species which possess the property of natural transformation [152]. The importance of transformation for genetic evolution in pneumococcal populations is illustrated by the emergence of penicillin-resistant pneumococcal isolates and pneumococcal strains undergoing capsule switch [103-105]. Possible sources of transforming DNA are other bacterial species sharing the same ecological niche, e.g. oral streptococci [153, 154] and DNA released from a subpopulation of pneumococcal cells during competence [36, 39].

In *S. pneumoniae*, competence is induced by the competence stimulating peptide (CSP) (for a review see [36]). Induction of competence-specific (*com*) genes leads to DNA uptake and its processing. Competence also triggers cell lysis and DNA release from a fraction of bacterial cells. Coordination in time and space between release and uptake of DNA by competent cells enhances transformation efficiency. Cell lysis and subsequent DNA release requires at least one bacteriocin system (CibABC) and three cell-wall hydrolases (CbpD, LytA, and LytC) [37-41].

However, Moscoso et al. [38] showed that extracellular DNA can be detected when pneumococcal cells do not develop competence (non-permissive conditions for competence) and deletion of *lytA* and *lytC* in a competence-deficient strain does not abolish DNA release. Therefore, other pathway(s) must exist that lead to DNA release in the early growth phase and coincident with competence.

S. pneumoniae produces ample amounts of hydrogen peroxide (H_2O_2). This leads to the inhibition of a variety of competitive microorganisms [63] and cytotoxicity for cultured alveolar cells [155]. Also, endogenously generated H_2O_2 contributes to a high frequency of point mutations [52]. Most oxidants induce mutations by oxidizing the guanine base, since guanine is particularly susceptible to oxygen radicals. Oxidized guanine base introduces point mutations by pairing with adenine or thymine, instead of cytosine. Therefore, most prominent base substitutions are G:C → A:T transitions followed by G:C → T:A transversions [53]. In pneumococcus, H_2O_2 is produced under rich and aerobic conditions by the enzyme pyruvate oxidase (SpxB), encoded by the *spxB* gene. Besides the production of H_2O_2 , SpxB decarboxylates pyruvate to acetyl phosphate plus CO_2 [156]. Spellerberg et al. [64] showed that *spxB*-deficient mutant exhibit reduced virulence for nasopharyngeal colonization, pneumonia and sepsis.

In this study, the role of SpxB and endogenously produced H₂O₂ in DNA release from *S. pneumoniae* strain D39 was investigated. It was shown that expression of *spxB* gene and production of H₂O₂ peaked during the early growth phase, which coincides with the time of competence. There was no evidence for a strong, direct toxic effect of H₂O₂ on bacterial cells, since addition of catalase reduced DNA release only slightly. However, *spxB* seems to play a role in DNA release and competence, since *spxB* knock-out mutant exhibited reduced DNA release and significantly lower transformation rates. This is currently further investigated in expression studies of competence genes.

MATERIALS AND METHODS

Bacterial strains and culture

Streptococcus pneumoniae strain D39 (serotype 2) [133], was kindly provided by Prof Jeffrey Weiser (University of Pennsylvania, Philadelphia, U.S.A) and strain R6 (spontaneous non-encapsulated derivative of D39) by Prof Philippe Moreillon (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland).

Bacteria were grown on Columbia sheep blood agar (CSBA) plates at 37°C in a 5% CO₂-enriched atmosphere. Liquid culture was performed in brain heart infusion (BHI, pH 7.4-7.5) (Becton Dickinson and Company, le Pont de Claix, France) broth containing 5% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany) in a water bath at 37°C without shaking. Transformation experiments were performed either in BHI broth supplemented with 5% FCS or in TSB competence medium (pH=8) [157]. Bacteria were stored at -80°C using Protect bacterial preservers (TSC, Heywood, U.K.). Optical density was measured at 600 nanometers (OD_{600nm}) using PerkinElmer™ Lambda-2 Spectrometer (PerkinElmer (Schweiz) AG, Schwerzenbach, Switzerland).

Hydrogen peroxide release assay

The release of H₂O₂ by *S. pneumoniae* was determined by a modification of the protocol provided in the Amplex® Red hydrogen peroxide/peroxidase assay kit instruction manual (Molecular Probes, Eugene, Oregon, U.S.A).

Overnight culture was 1:100 diluted, incubated at 37°C and samples of 2.5 ml were withdrawn at OD_{600nm} 0.05, 0.15, 0.25, 0.35, 0.45, 0.55, 0.65 and 0.75. Samples were pelleted at 5000 rpm, 4°C for 10 minutes and washed with Hanks medium (4°C) (Gibco™ Hanks' Balanced Salt Solution (no phenol red), Invitrogen, Carlsbad, California, U.S.A). After an additional centrifugation the pellet was dissolved and 20-fold diluted in BHI with 5% FCS. 50 µl Hanks medium containing 0.2 U/ml horseradish peroxidase and 100 µM Amplex Red reagent were added to 50 µl bacterial culture. Standard curves were generated with fresh dilutions of 30% hydrogen peroxide. The reactions were incubated at 37°C, and the absorbance was read at 563 nm (SpectraMax GeminiXS, Molecular Devices, Sunnyvale, California, U.S.A) every minute for 15 minutes. In parallel, serial dilutions of the same bacterial culture were spread onto CSBA plates to determine the total cell count. The H₂O₂

release in μM per hour and 10^6 colony forming units (cfu) were calculated. For each experiment three independent measurements were performed on different days.

RNA isolation for RT-PCR

Bacteria were grown in BHI with 5% FCS to an $\text{OD}_{600\text{nm}}$ of 0.05, 0.15, 0.25, 0.35, 0.45, 0.55 or 0.65, twice the culture volume of RNAprotect™ (Qiagen AG, Hombrechtikon, Switzerland) was added to prevent further transcription and to prevent RNA degradation. After vortexing and a 5 min incubation at room temperature (RT), the bacteria were pelleted by centrifugation for 10 min at 5000 g. The pellet was resuspended in 200 μl TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH=8) containing 600 μg lysozyme (Roche Diagnostics GmbH, Mannheim, Germany). After 10 min at RT with repeated vortexing, 700 μl RLT buffer (Qiagen RNeasy® Mini kit), containing β 2-mercaptoethanol (Merck KGaA, Darmstadt, Germany), was added and the tubes vortexed. The mixture was transferred to a 1.5 ml tube containing 0.05 g 100 μm acid-washed glass beads (Sigma-Aldrich, Buchs, Switzerland) and vibrated for 10 min at half maximum speed using a Mickle Vibratory Tissue Disintegrator (Mickle Laboratory Engineering, Gomshall, U.K.). The mixture was then centrifuged and RNA extracted from the supernatant using Qiagen RNeasy® Mini kit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer's instructions. The RNA recovered was treated with DnaseI (Stratagene Europe, Amsterdam, Netherlands) according to the manufacturer's instructions to remove any contaminating DNA. RNA concentration and purity were determined by measuring absorbance at both 260 nm and 280 nm (Lambda-2 Spectrometer, PerkinElmer (Schweiz), Schwerzenbach, Switzerland).

Quantitative gene expression using a cRNA standard curve

Quantitation of absolute mRNA copy numbers by real-time RT-PCR was performed by using a standard curve generated based on *in vitro* transcribed RNA (cRNA) as previously described [148, 149].

The primer pair for the standard curve was designed to flank the real-time PCR target sequence of *spxB* gene (spxB_F1_T7: 5'-TAATACGACTCACTATAGGGAGAGTGGAATAGTAAAAATTTGGAGAACG-3', spxB_Bac1: 5'-CGATCTTTTAAAGTTCTGCTCTATG-3'). The forward primer contained the T7 RNA polymerase promoter (T7 promoter is underlined) sequence at

the 5' end to allow *in vitro* transcription from PCR product using the MEGAscript T7 High Yield Transcription Kit (Ambion, Cambridgeshire, U.K.) followed by treatment with DNase I (Stratagene Europe, Amsterdam, Netherlands) according to manufacturer's protocol. Standard curve cRNA was purified using the RNeasy Kit (Qiagen AG, Hombrechtikon, Switzerland) according to the RNA clean-up protocol supplied by the manufacturer. Size and integrity of transcripts was verified on a 1.2% formaldehyde gel. cRNA was quantified by spectrophotometry at 260 nm (Lambda-2 Spectrometer, PerkinElmer (Schweiz), Schwerzenbach, Switzerland). Concentration of cRNA was converted into copy number according to the following equation: N (copies/ μ l) = C (cRNA in μ g/ μ l) / K (fragment size in bp) * 182.5×10^{12} (Avogadro constant). 3 μ l standard curve cRNA of serial ten-fold dilutions (10^{12} to 10^0 copies/ μ l) and 3 μ l RNA extracted from 10 ml bacterial culture of OD_{600nm} 0.05, 0.15, 0.25, 0.35, 0.45, 0.55 and 0.65 was reversed transcribed to cDNA using Superscript II (Amersham, Buckinghamshire, U.K.) and random hexamer primers (Promega, Wallisellen, Switzerland) according to supplier's protocol. For the cultures of each OD_{600nm} from which we extracted RNA, serial dilutions were spread onto CSBA plates to determine the cell count (cfu). Quantification of gene expression was achieved by real time RT-PCR using TaqMan primers and probes created by the Assay-by-Design™ Service of Applied Biosystems (Rotkreuz, Switzerland) based on the most conserved regions of the *spxB* gene in *S. pneumoniae* strain TIGR4 (AE005672), strain R6 (AE008442), strain D39 (AY254852) and strain AB15 (AY254854). (forward primer *spxB*-tgt3F: 5'- ACAGGTTCTGCTTACCGTGTTG-3', reverse primer *spxB*-tgt3R: 5'- AGGAAAAGAACTGTGTCTGCTTCAA-3' and probe *spxB*-tgt3M2: 6-FAM- TCGTTGGCTGGTTTCCAA-MGB). cDNA was diluted 4-fold in the assay and an RT-negative control was performed for every sample. Real-time RT-PCR was performed in 96-well plates using ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). The experiment was performed on three different days and real-time RT-PCR was carried out in triplicates.

Finally, for the standard curve, copy number was plotted against cycle-of-threshold (Ct) value (Applied Biosystems, ABI PRISM SDS 7000 Software v1.1) and the Ct values and the cell count of the extracted RNA cultures were used to calculate the copy number of the samples per 10^2 cfu.

Transformation assay for DNA release

The release of DNA into the culture medium was assayed by transformation of competent cells of a Sm^S recipient strain R6 *hexA* mutant with cell-free filtrates from D39 Sm^R strain or its *spxB* mutant cultures as previously described [38].

To select a D39 resistant mutant, bacteria were grown overnight in BHI with 5% FCS. 100 µl of the O/N culture was diluted 50-fold in fresh BHI with 5% FCS. Diluted culture was grown at 37°C to an OD_{600nm} of 0.8 and 200 µl were plated on CSBA plates containing 300 µg/ml streptomycin. Streptomycin-resistant strain D39 (D39 Sm^R) was selected after 24 hours of incubation at 37°C in a 5% CO₂-enriched atmosphere.

To delete *hexA* in R6, a 2.6 kb fragment of *hexA* gene of strain 108.21 (non-encapsulated *S. pneumoniae*) was amplified (primers: *hexA_f11*: 5'-AGAGACAGAAAATGGCGATAGAAA-3', *hexA_b2641*: 5'-ATAGACAAAAGGGAGCGACAATG-3') and cloned into pGEM[®]-T Easy Vector (Promega, Wallisellen, Switzerland). A 1000 bp fragment of *hexA* was cut out by HindIII and was replaced with an erythromycin cassette (*ermB* of pJDC9). *S. pneumoniae* strain R6 was transformed with the whole plasmid and recombinants were selected on CSBA plates containing erythromycin (2 µg/ml). Positive recombinants were analysed by Southern blot, PCR and phenotypically a 10-fold higher mutation rate to rifampicin resistance compared to the wild-type was revealed (data not shown).

An overnight culture of strain D39 Sm^R or its *spxB* mutant was prepared with 3 to 10 colonies in 5 ml BHI broth containing 5% FCS. 100 µl of the overnight culture was subcultured in 5 ml BHI with 5% FCS and grown to mid-log phase (OD_{600nm} 0.5). Two ml mid-log culture was pelleted at 3800 rpm, 4°C for 10 minutes and washed with 5 ml Hanks medium to remove any extracellular DNA. After repeating the wash step with Hanks medium, the pellet was diluted 100-fold in BHI with 5% FCS and 20 ml were incubated at 37°C. If stated 5000 U/ml catalase (Sigma-Aldrich, Buchs, Switzerland), 0.1% sodium acetate (Merck, Darmstadt, Germany) and/or H₂O₂ (see below) was added to the BHI broth with 5% FCS.

Samples of 0.2 ml were withdrawn at OD_{600nm} 0.05, 0.15, 0.25, 0.35, 0.45, 0.55, 0.65 and 0.75. Cells were then removed by filtration and centrifugation for 60 seconds, using Micropure[®]-EZ enzyme removers (Millipore AG, Volketswil, Switzerland). To maximize DNA recovery, 50 µl TE buffer (pH=8.0) was added to Micropure-EZ

reservoir and spun 30 seconds. Cell-free filtrates were stored 1-2 days at -20°C until the transformation assay.

Competent cells of strain R6 *hexA* mutant were prepared by inoculating BHI with 5% FCS with 3 to 10 colonies and allowed to grow overnight. A fresh culture was started in the morning by diluting the overnight culture 1:100 in fresh broth and was grown to an $\text{OD}_{600\text{nm}}$ of 0.15. The culture was diluted 1:20 in BHI broth with 5% FCS or TSB competence 8.0 medium prewarmed at 30°C , and aliquots of 750 μl were incubated for 15 min. Competence stimulating peptide (CSP-1) (Neosystems, Strasbourg, France) was added to a final concentration of 100 ng/ml, and incubated for 15 min at 30°C . 250 μl of withdrawn cell-free filtrates were added and incubated for 40 minutes at 30°C and then for 90 minutes at 37°C . Aliquots of 200 μl were spread in duplicates on CSBA plates containing 200 $\mu\text{g/ml}$ streptomycin, incubated for 24 hours before counting the number of Sm^{R} transformants. For each experiment three independent measurements were performed on different days.

Construction of D39 *spxB* mutant

Transformation of *Escherichia coli* and *S. pneumoniae* was performed as described previously [125, 127].

A 1.3 kb fragment (*spxB*_S2_F: 5'-TAAATTCGGCGGCTCAATC-3', *spxB*_S1_B: 5'-CAGCGTTTGTGAAGTCTACACC-3') of *spxB* was amplified and cloned into pGEM[®]-T Easy Vector (Promega, Wallisellen, Switzerland). An erythromycin cassette (*ermB*) [136] was inserted at the HindIII restriction site of the amplified *spxB* fragment. Strain D39 Sm^{R} was transformed with the whole plasmid and recombinants were selected on CSBA plates containing erythromycin (2 $\mu\text{g/ml}$). Knock-out of *spxB* was confirmed by PCR and phenotypically by a 4-fold decreased H_2O_2 release per cell and a 10-fold decreased H_2O_2 concentration in the supernatant at $\text{OD}_{600\text{nm}}$ 0.75 compared to the wild-type (data not shown).

Supplementation of culture medium of mutant D39 Sm^R ΔspxB with H₂O₂

In order to complement *spxB*-deficient mutant with a physiological concentration of H₂O₂, the accumulation of H₂O₂ in the supernatant of strain D39 Sm^R was first determined.

An overnight culture was 1:50 diluted in BHI with 5% FCS and was grown to mid-log phase (OD_{600nm} 0.5). Cells from 2 ml of the mid-log culture were pelleted at 3800 rpm, 4°C for 10 minutes and washed twice with 5 ml Hanks medium. The pellet was then diluted 100-fold in BHI with 5% FCS and incubated at 37°C. Samples of 0.2 ml were withdrawn at different OD_{600nm}. The H₂O₂ concentration in the supernatant was measured by using the Amplex[®] Red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Eugene, Oregon, U.S.A). 50 µl bacterial culture was applied to a 96-well plate (Nunclon[™] Nalge Nunc, Roskilde, Denmark) and 50 µl Hanks medium containing 0.2 U/ml horseradish peroxidase and 100 µM Amplex Red reagent were added. The absorbance was read at a wavelength of 563 nm (SpectraMax GeminiXS, Molecular Devices, Sunnyvale, California, USA). Concentrations were determined based on a standard. The concentrations of H₂O₂ in the supernatant of D39 Sm^R at OD_{600nm} 0.005, 0.05, 0.15, 0.25, 0.45 and 0.75 were 1.5, 150, 270, 320, 525 and 480 µM respectively.

To mimic natural H₂O₂ concentrations in the growth medium, the *spxB*-deficient mutant was grown in 20 ml BHI with 5% FCS. At the start 0.05 µmoles H₂O₂ (Merck, Darmstadt, Germany) was added to reach an initial concentration of 2.5 µM. Thereafter, H₂O₂ was added at intervals of 10 minute for a total of 300 minutes. The amount of H₂O₂ added was incrementally increased by 0.05 µmoles for each subsequent addition (i.e. 0.05, 0.1, 0.15, 0.2 µmoles etc).

Assay for transformation rates

Transformations were performed as described above with the following differences: Rifampicin susceptible strain D39 Sm^R or its *spxB*-deficient mutant were transformed with a total of 1 µg DNA consisting of the rifampicin resistant (Rif^R) *rpoB* gene as previously described [125]. Transformation was performed with and without addition of CSP-1. Culture of 100 µl were spread on CSBA plates containing 0.5 µg/ml rifampicin. The plates were incubated for 48 hours before counting the transformants. In parallel, serial dilutions of the same culture were spread onto CSBA plates to determine the total cell count.

Statistical analyses

Statistical analyses were done in StatView® version 5.0 (SAS Institute Inc., Cary, NC). Proportions were compared with the Chi square test or Fisher's exact test as appropriate. Differences between means were assessed by the Student's t-test. A cut-off of $p \leq 0.05$, two tailed, was used for all statistical analyses.

RESULTS

*Kinetics of H₂O₂ release and *spxB* expression during growth*

This experiment investigated whether H₂O₂ release and/or *spxB* gene expression depends on the growth phase. It could be shown that H₂O₂ release peaked significantly at an OD_{600nm} of 0.25 ($p < 0.05$ compared to all other optical densities) (Fig. 1). Also, *spxB* transcription was significantly upregulated at OD_{600nm} 0.150 ($p < 0.05$ compared to all other optical densities) (Fig. 2).

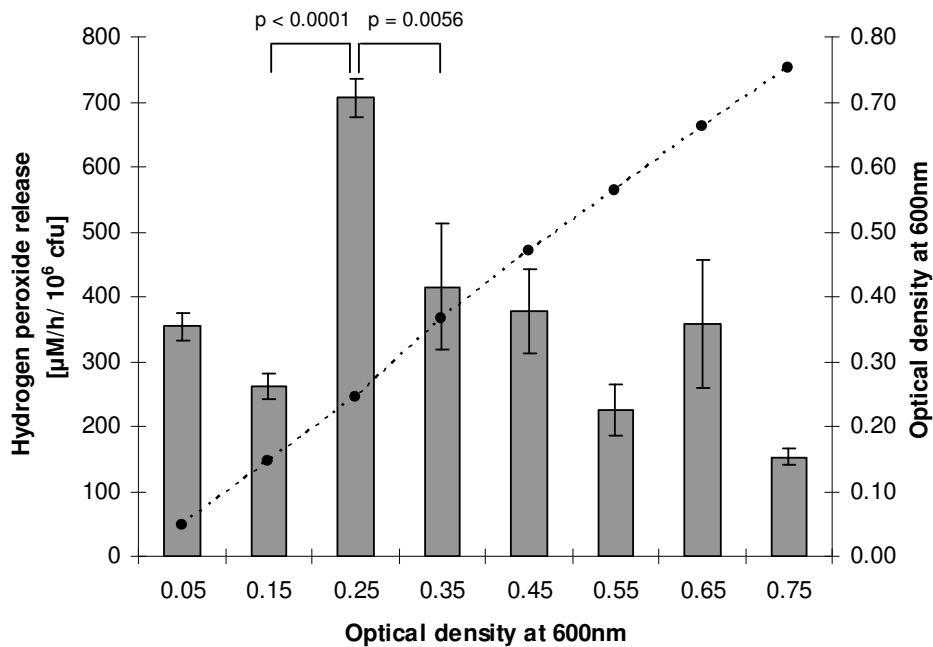


Figure 1. Hydrogen peroxide release of strain D39 during the lag and log phase.

Left axis: The H₂O₂ release of strain D39 was measured in BHI with 5% FCS and the release per 10⁶ colony forming units were calculated. Presented are mean values of duplicates from three independent experiments (\pm SE). **Right axis:** Presented are the mean values of the optical densities obtained during the 3 independent experiments.

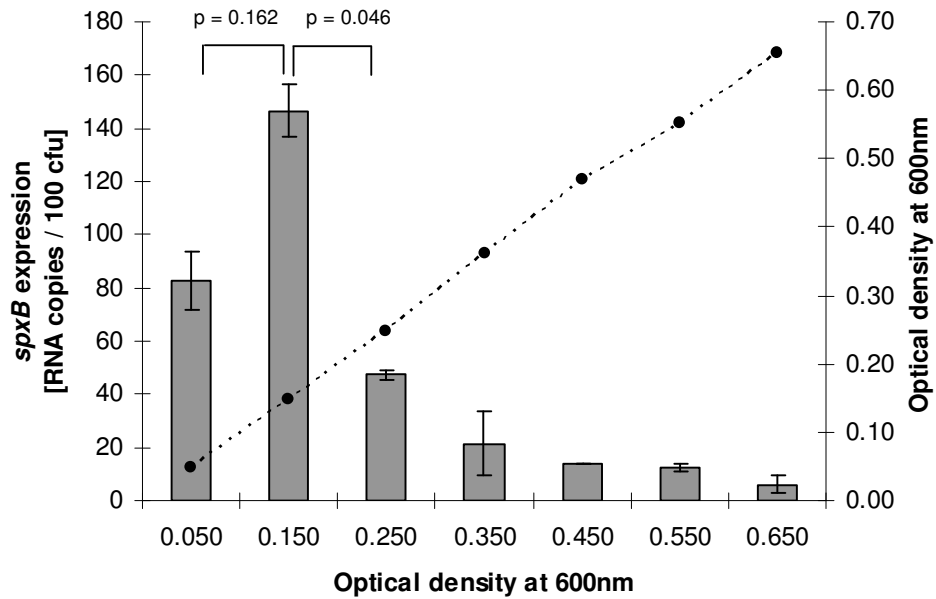


Figure 2. *SpxB* gene expression of strain D39 during the lag and log phase.

Left axis: The *spxB* transcription levels were determined by real-time RT-PCR expressed as the copy number per 100 cfu. Presented are mean values of triplicates from three independent experiments (\pm SE). **Right axis:** Presented are the mean values of the optical densities at 600nm obtained during the 3 independent experiments.

Influence of *spxB* gene on DNA release

DNA release was investigated in D39 Sm^R and its isogenic *spxB* mutant. In the parental strain D39 Sm^R the liberation of DNA peaked at OD_{600nm} 0.15 and decreased thereafter (Fig. 3). The *spxB* mutant showed significantly reduced DNA release during the lag and the early-log phase. The difference in DNA release between D39 Sm^R and its *spxB* mutant was greatest (10-fold) at OD_{600nm} 0.15 (p=0.0004).

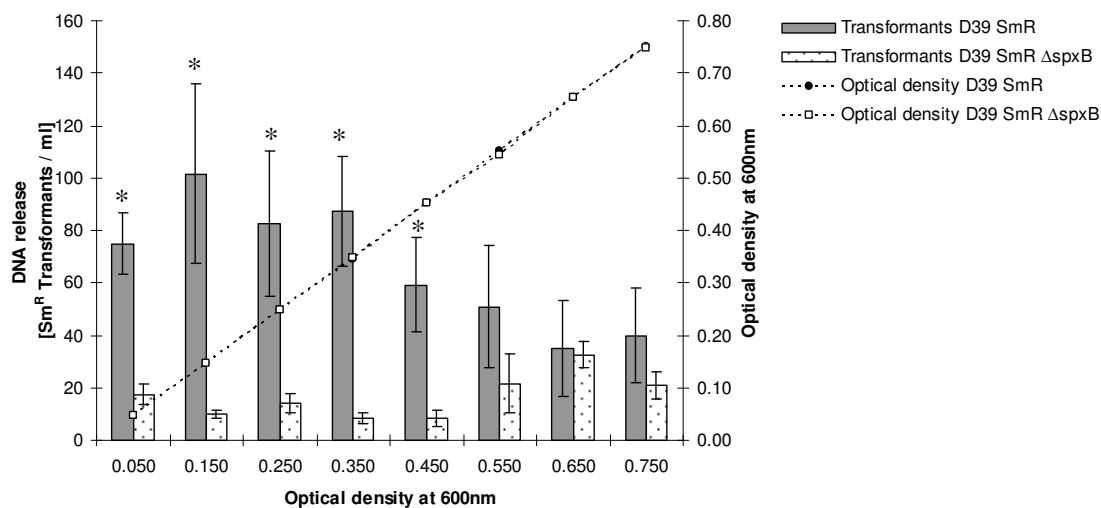


Figure 3. DNA release of strain D39 Sm^R and its *spxB* mutant during the lag and log phase.

Left axis: Values for DNA release represent streptomycin-resistant transformants per ml. Presented are mean values of duplicates from three independent experiments (\pm SE). * p<0.05; the p-values were calculated by comparing the DNA release of D39 Sm^R with the DNA release of D39 Sm^R Δ*spxB* at each optical density. **Right axis:** Presented are the mean values of the optical densities at 600nm obtained during the 3 independent experiments.

Influence of catalase supplementation on DNA release in strain D39 Sm^R

In order to test whether reduction of DNA release in *spxB* mutant was mediated by H₂O₂, DNA release in parental strain D39 Sm^R was measured in the presence of catalase. The addition of catalase reduced the H₂O₂ concentration in the supernatant of strain D39 Sm^R to an undetectable level (data not shown). There was a trend towards reduced DNA release in the early growth phase (OD_{600nm} 0.025 and 0.05) in the presence of catalase. The difference to cultures without catalase was statistically significant, when the data from OD_{600nm} 0.025 and 0.05 were taken together (p=0.016).

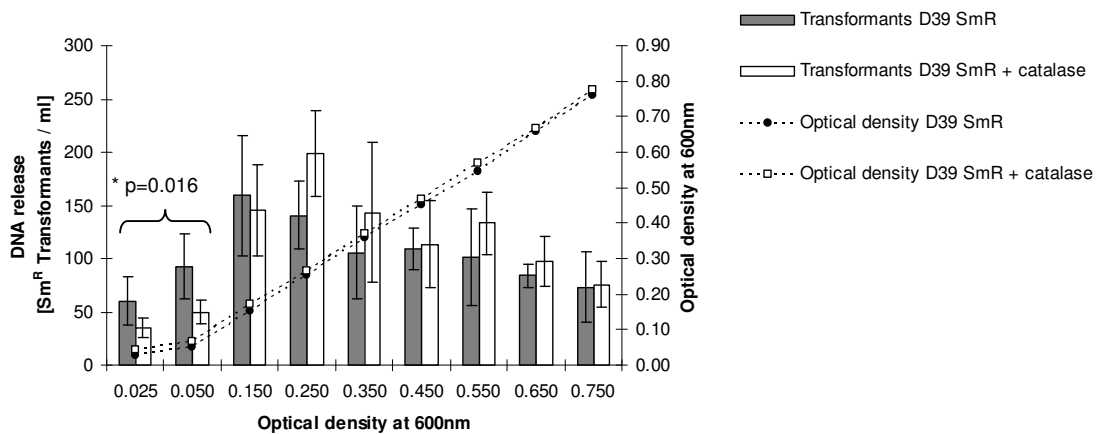


Figure 4. Influence of catalase on DNA release of strain D39 Sm^R during the lag and log phase.

Left axis: Values for DNA release represent streptomycin-resistant transformants per ml. Presented are mean values of duplicates from three independent experiments (\pm SE). * The data from OD_{600nm} 0.025 and 0.05 were taken together and the p-value was calculated by comparing the DNA release in the presence of catalase with the DNA release measured without catalase supplementation. **Right axis:** Presented are the mean values of the optical densities at 600nm obtained during the 3 independent experiments.

Influence of acetate and/or H₂O₂ supplementation on DNA release in *spxB*-deficient mutant

In order to investigate whether H₂O₂ did act as a messenger for DNA release alone or in combination with acetyl phosphate (another end product of SpxB), DNA release was measured in the *spxB* mutant with H₂O₂ supplementation and/or the addition of acetate [64]. Addition of H₂O₂ or sodium acetate or both did not influence DNA release significantly (Fig. 5).

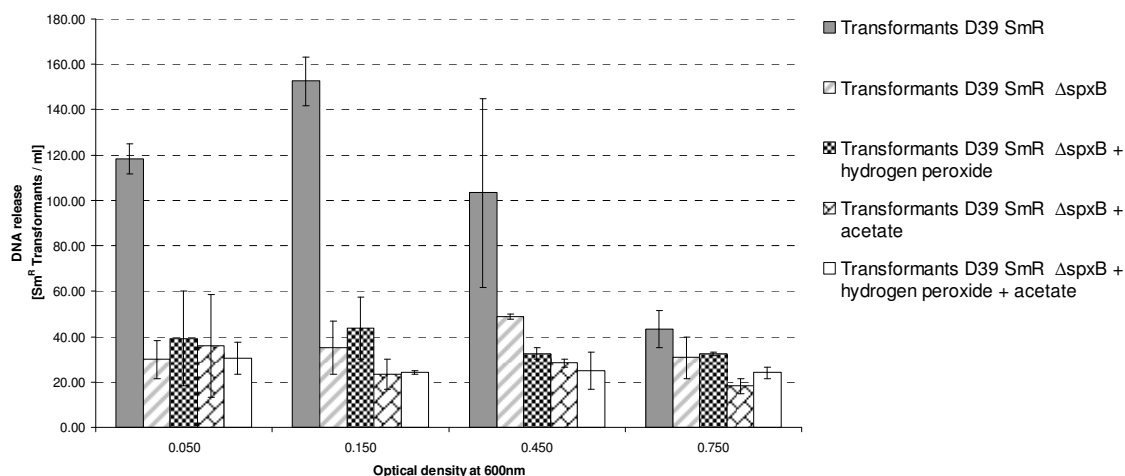


Figure 5. DNA release of *spxB*-deficient mutant grown in culture medium supplemented with acetate and/or H₂O₂. Left axis: Values for DNA release represent streptomycin-resistant transformants per ml. Presented are mean values of triplicates from three independent experiments (\pm SE). **Right axis:** Presented are the mean values of the optical densities at 600nm obtained during the 3 independent experiments.

Influence of *spxB* gene on transformation

Transformation efficiency of *spxB*-deficient mutant to rifampicin resistance with or without addition of CSP-1 was measured and compared to its parent strain D39 Sm^R. No significant difference ($p=0.68$) in the transformation rate was shown between the *spxB* mutant (mean $1.8 \cdot 10^{-2}$, SE $7 \cdot 10^{-2}$) compared with D39 Sm^R (mean $2.3 \cdot 10^{-2}$, SE $6 \cdot 10^{-2}$) in the presence of CSP-1. As expected, lack of CSP-1 supplementation decreased transformation efficiency significantly in the parent strains and its isogenic *spxB* mutant ($p=0.031$ and $p=0.045$ respectively). However, while transformants could still be recovered from the parent strain at a mean frequency of $6.3 \cdot 10^{-6}$ (SE $1.6 \cdot 10^{-6}$), no transformants were obtained from the *spxB* mutant. This corresponds to a 10^4 times lower transformation rate in strain D39 Sm^R in the absence of CSP-1.

DISCUSSION

S. pneumoniae produces substantial amounts of H₂O₂ (in vitro up to 1-2 mM) [52, 63, 156], which exhibits toxicity for competing microorganisms and human cells [63, 155, 158]. Nevertheless, *S. pneumoniae* does not harbor a catalase and is therefore exposed to its own toxic product [52]. This study investigated, whether pyruvate oxidase (SpxB) and its product, H₂O₂, play a role in DNA release from bacterial cells during the early growth phase [36-39].

It was found that H₂O₂ production peaked during the early growth phase, which coincides with the time of competence. The amounts of H₂O₂ produced were up to 4.5-fold greater at an OD₆₀₀ of 0.25 than during earlier or later phases of growth. Expression studies of *spxB*, also described in chapter 3, showed that H₂O₂ production was likely controlled at the transcriptional level, since *spxB* gene expression also peaked in the early growth phase. This corresponds to the observation of Lee et al. [150], who showed an induction of pyruvate oxidase (SpxB) in the early growth phase using 2-D gel electrophoresis.

Interestingly, deletion of *spxB* gene led to a significant reduction of DNA release. Therefore, *spxB* seems to be involved in DNA release but the mechanism by which this occurs is not clear. *SpxB* encodes the enzyme pyruvate oxidase, which produces H₂O₂, acetyl phosphate and CO₂ by decarboxylation of pyruvate. Results of Belanger et al. point to a role of endogenously produced H₂O₂ on bacterial cell death in the center of R36A and R6 colonies grown on agar plates [133]. In addition, the red blood cells incorporated into agar plates used for growth of *S. pneumoniae* are thought to counteract the negative effects of H₂O₂. Alternatively, exogenous catalase may be added to the growth medium instead of blood to facilitate bacterial growth.

Supplementation of catalase reduced the DNA release only slightly during the early growth phase. A strong direct toxic effect of H₂O₂ on bacterial cells, which leads to DNA release seems therefore unlikely. However, catalase may not have inactivated intracellular H₂O₂, since the enzyme cannot penetrate cell membranes in contrast to H₂O₂. Ongoing H₂O₂ production may provide low concentrations of H₂O₂ within pneumococcal cells despite the presence of catalase. This low concentration may not be toxic, since toxic effects of H₂O₂ on bacterial cells are reported in mM concentrations [63, 156, 159]. Inefficient supplementation of catalase was not responsible for the limited reduction of DNA release during the early growth phase. Addition of 5000 U/ml of catalase was sufficient to keep the H₂O₂ concentration to an

undetectable level during the whole experiment, but had no further effect on DNA release than lower amounts of catalase (data not shown).

Based on our results, we hypothesized that the effect of *spxB* deletion may be explained by a role for H₂O₂ alone, or in combination with acetyl phosphate, as an intracellular messenger (rather than the toxic effect of H₂O₂) (Fig. 6). However, supplementation of cell cultures with H₂O₂ and/or acetate did not restore DNA release in an *spxB* mutant. The hypothesis appears attractive nevertheless, since there is ample evidence that acetyl phosphate can act as intracellular messenger. Kim et al. [160] demonstrated that in a *comP* mutant of *Bacillus subtilis* (ComP is equivalent to ComD in *S. pneumoniae*), ComA can be activated by acetyl phosphate. ComA is equivalent to ComE in *S. pneumoniae*. ComE~P is responsible for activation of the competence regulon, which maintains and regulates the whole machinery of DNA uptake and its processing [36]. In pneumococcus, Spellerberg et al. [64] demonstrated that addition of 0.1% sodium acetate (which restores the acetyl phosphate level) restored the adherence deficiency of a *spxB* mutant.

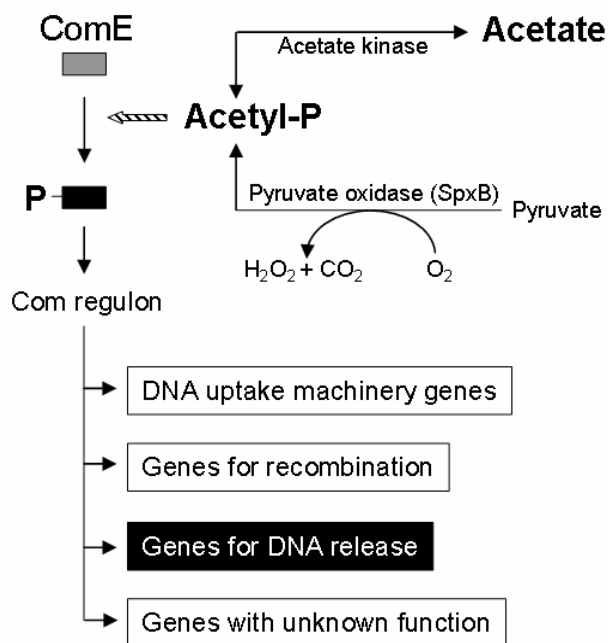


Figure 6. Model for the influence of SpxB on DNA release. Acetyl phosphate (Acetyl-P), the endproduct of pyruvate oxidase (SpxB), acts as phosphor-donor to phosphorylate ComE. ComE~P upregulates the competence regulon (Com regulon).

The *com* regulon encodes multiple genes and induces, via complex pathways, the machinery for DNA uptake, its processing and DNA release from a subfraction of bacterial cells.

This study showed that natural transformation was abolished in the *spxB*-deficient mutant. There was no difference in the transformability between an *spxB* mutant and the wild-type when the competence stimulating peptide (CSP) was added. This indicates that deletion of *spxB* did not affect the response to CSP, DNA uptake or recombination. In addition, Peterson et al. showed that expression of *spxB* is not controlled by CSP [161, 162]. The fact that *spxB* gene influences transformation in the absence of CSP, points to a link between *spxB* and the competence machinery. Trombe et al. [163] demonstrated that deletion of *nox* gene (encoding for NADH oxidase) reduced the transformability by 50%. Interestingly, NADH oxidase influences competence by the activation of ComE, as we speculated for SpxB. NADH oxidase, as pyruvate oxidase (SpxB), utilizes O₂. In addition, competence development in *Streptococcus pneumoniae* is strongly dependent on the availability of oxygen in cultures [163]. Trombe et al. [163-166] hypothesized that NADH is a sensor for oxygen and that competence is regulated by O₂.

In conclusion, the pneumococcal *spxB* gene plays a role for the competence associated DNA release from a subfraction of bacterial cells. There is no evidence that *spxB* mediates this effect through the toxic effect of H₂O₂. The role of *spxB* products as intracellular messengers is an attractive explanation. However, no evidence for such a pathway was found in this study. To investigate the influence of *spxB* gene on competence further, gene expression of an early- (*comC*) and late-com gene (*recA*) is currently being performed. The observation that the *spxB* gene influences DNA release is intriguing. Quorum sensing is no longer believed to be for competence [36]. SpxB may be an attractive candidate.

ACKNOWLEDGMENTS

This study was supported by a grant from the Swiss National Science Foundation to K.M., Grant No 3200-067998. We thank Suzanne Aebi for her laboratory support and Sidi Christen for technical support and fruitful discussions.

CHAPTER 5

Serotype-specific invasiveness and colonization prevalence in *Streptococcus pneumoniae* correlate with the lag phase during in vitro growth

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This study has been published in *Microbes and Infection*. 2006; 8:2612-2617.

CHAPTER 6

Role of pneumococcal capsule genes in growth

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The chapter will be submitted as a note for publication.

Data were presented at the ASM Conference on Streptococcal Genetics, St. Malo, France, 18 to 21 June 2006.

SUMMARY

The polysaccharide capsule is the major virulence factor of the important human pathogen *Streptococcus pneumoniae*. Serotypes differ for invasive potential and colonization prevalence for unknown reason. Recently, we showed that there is a serotype-specific growth behavior. This study investigates whether capsule genes influence bacterial growth in vitro.

Deletion of the whole capsule operon in D39 (strain D39::cpsA-O) led to a significantly prolonged lag phase in THY broth compared to the parent strain D39 ($p=0.0002$). Backtransformation of the capsule operon into D39::cpsA-O (strain D39::cpsA-O::S2) restored growth. Partial deletions of the capsule operon showed different influence on growth behavior. Significantly prolonged lag phase was observed in strains D39::cpsK-O and D39::cpsM-O ($p<0.0001$ for both mutants). Whereas mutant strains D39::cpsF-O, D39::cpsL-O, D39::cpsN-O and D39::cpsO showed no change in growth parameters. Supplementation of THY with 5% FCS restored growth of capsule operon-deficient mutant and at least partially in those partial capsule operon mutants with a prolonged lag phase.

In conclusion, this study showed that deletion of pneumococcal capsule genes in D39 influenced the lag phase in bacterial growth. Capsule gene products may have multiple functions in the bacterial metabolism besides their role in polysaccharide biosynthesis.

INTRODUCTION

The polysaccharide capsule is the most important virulence factor of pneumococcus and non-encapsulated pneumococci are of low pathogenicity [167]. At least 90 different polysaccharide types have been identified [83, 118]. Serotypes differ for invasive potential and colonization prevalence [107, 108]. Recently, we investigated 47 clinical pneumococcal isolates, representing 15 different serotypes, and showed that there is a serotype-specific growth behavior during in vitro growth in unsupplemented brain heart infusion broth [129]. Intriguingly, serotypes with high invasive potential had a longer lag phase and serotypes associated more with colonization than invasiveness had a shorter lag phase.

Each serotype consists of structurally distinct capsular polysaccharide composed of repeating oligosaccharide units joined by glycosidic linkages [118]. Genes required for the polysaccharide capsule synthesis are located in an operon flanked by *dexB* and *aliA* for most serotypes [97, 168]. Capsule operons of different serotypes show a similar structure with some conservation particularly within the first four genes, downstream of which are the serotype-specific genes [97, 118, 168]. The first four genes, *cpsA-D*, play a role in regulation of capsule expression [169, 170]. For example, the serotype 2 capsule operon of D39 consists of 17 capsule genes (*cpsA-B-C-D-E-T-F-G-H-I-J-K-P-L-M-N-O*) [88]. The first four genes (*cpsA-B-C-D*) are involved in regulation and export of the CPS. The remaining genes encode for glycosyltransferases (*cpsE-T-F-G-I*), a CPS polymerase (*cpsH*), a CPS repeat unit flippase (*cpsJ*), a UDP-glucose-dehydrogenase (Ugd, *cpsK*), a galactopyranose mutase (*cpsP*, unique to the type 2 capsule) and genes involved in rhamnose production (*cpsL-M-N-O*). Most of the carbohydrates required for the polysaccharide synthesis are encoded by the capsule operon, but some precursors that occur also in other cellular structures, such as glucose, N-acetylglucosamine (of peptidoglycan), and ribitol (of the teichoic acids), are catalyzed by enzymes encoded by genes located outside the capsule operon.

Previous studies have shown that deletion of genes involved in the production of polysaccharide capsule in *Cryptococcus neoformans* or *Streptococcus mutans* not only disrupted capsule production, but affected other cellular characteristics such as colony morphology, temperature sensitivity, growth, and sensitivity to the nutritional environment [119-121]. Also, in *C. albicans* glycosyltransferases (enzymes involved in polysaccharide biosynthesis) can have multiple cellular functions [122]. These

findings suggest, that capsule genes may have multiple functions in the bacterial metabolism besides the biosynthesis of a polysaccharide capsule.

In this study, we investigate whether the in vitro growth of pneumococcal strain D39 (serotype 2) is influenced by its capsule genes. It was found that deletion of the whole capsule operon led to a significantly prolonged lag phase compared to the parent strain D39. Mutants of D39 with partial deletion of the capsule operon showed different growth behavior. FCS supplementation (fully) compensated the prolonged lag phase of capsule gene-deficient mutants.

MATERIALS AND METHODS

Bacterial strains

Streptococcus pneumoniae strain D39 (serotype 2) [133] was kindly provided by Prof Jeffrey Weiser (University of Pennsylvania, Philadelphia, U.S.A). Capsular serotype was analyzed by the Quellung reaction as previously described [9, 126] or the “agglutination reaction” (Statens Serum Institut, Copenhagen, Denmark).

Construction of D39 capsule mutant (D39::cpsA-O)

Transformation of *Escherichia coli* and *S. pneumoniae* was performed as described previously [125, 127]. The plasmid pBluescript KSII (Stratagene Europe, Basel, Switzerland) was used for plasmid construction.

Within pBluescript KSII the following genes were cloned into its multiple cloning site. First, a ~1000 bp fragment of the *dexB* ORF of strain D39, flanked by an erythromycin cassette (*ermB*) from the plasmid pJDC9 [136] was inserted. Downstream of *ermB* a ~1000 bp fragment of the *aliA* ORF of strain D39 was added. The whole *dexB-ermB-aliA* construct (~3000 bp) was amplified by PCR and used to transform competent cells of *S. pneumoniae* strain D39. Positive recombinants, selected on Columbia sheep blood agar (CSBA) plates containing erythromycin (2 µg/ml), were analysed by PCR and sequencing to confirm correct deletion of open-reading frames (ORF) *cpsA* to *cpsO*.

Backtransformation of D39 capsule operon into D39::cpsA-O (D39::cpsA-O::S2)

The chloramphenicol acetyltransferase (*cat*) antibiotic cassette of pJS3oG7 [126] was cloned between *cpsO* and *aliA* ORF of strain D39 (strain D39 cap::cat). Then strain D39::cpsA-O was transformed with the chromosomal DNA of D39 harboring the chloramphenicol cassette and recombinants were selected on CSBA plates containing chloramphenicol (3 µg/ml). All transformants had a smooth colony appearance and were of serotype 2. PCR results confirmed the insertion of the serotype 2 capsule between *dexB* and *aliA*.

Construction of D39 partial capsule mutants (Table 1)

The following genes were cloned into the multiple cloning site of pBluescript KSII. A ~1000 bp fragment downstream of *aliA* (*down_aliA*) ORF of strain D39 was inserted. Upstream of this fragment a ~1000 bp fragment of *cpsT*, *cpsJ*, *cpsP*, *cpsL*, *cpsM* or

cpsN ORF, respectively, of strain D39 was cloned. Between these two recombination regions, at an *EcoRI* restriction site, an erythromycin cassette (*ermB*) was inserted. *S. pneumoniae* was transformed with the whole plasmid containing the construct *cpsT*, *J*, *P*, *L*, *M* or *N-ermB-down_aliA*, respectively. Recombinant clones were selected on CSBA plates containing erythromycin (2 µg/ml). Positive recombinants were analysed by PCR and sequencing to confirm correct deletion of *cpsF-O*, *cpsK-O*, *cpsL-O*, *cpsM-O*, *cpsN-O* or *cpsO* ORFs, respectively. All mutants were non-typable by agglutination.

Construction of D39 *aliA* mutant (D39::*aliA*)

A 1.3 kb fragment of *aliA* ORF of strain D39 was amplified and cloned into pGEM[®]-T Easy Vector (Promega, Wallisellen, Switzerland). At the *HindIII* restriction site of the *aliA* fragment an erythromycin cassette was inserted. *S. pneumoniae* was transformed with the whole plasmid and recombinants were selected on CSBA plates containing erythromycin (2 µg/ml). Confirmation of the D39 *aliA* mutant was done by PCR and sequencing.

Table 1. Pneumococcal laboratory strains and mutants used in this study

Strain	Genotype and construction ^a	Serotype	Reference
D39	Erm ^S , Cm ^S	2	[133]
D39 <i>cap::cat</i>	D39 with chloramphenicol cassette inserted between <i>cpsO</i> and <i>aliA</i> ORF, Cm ^R	2	this study
D39:: <i>cpsA-O</i>	D39 Δ <i>cpsA-O</i> , Erm ^R	nt	this study
D39:: <i>cpsA-O::S2</i>	D39:: <i>cpsA-O</i> transformed with chromosomal DNA of D39 <i>cap::cat</i> , Cm ^R	2	this study
D39:: <i>cpsF-O</i>	D39 Δ <i>cpsF-O</i> , Δ <i>aliA</i> , Erm ^R	nt	this study
D39:: <i>cpsK-O</i>	D39 Δ <i>cpsK-O</i> , Δ <i>aliA</i> , Erm ^R	nt	this study
D39:: <i>cpsL-O</i>	D39 Δ <i>cpsL-O</i> , Δ <i>aliA</i> , Erm ^R	nt	this study
D39:: <i>cpsM-O</i>	D39 Δ <i>cpsM-O</i> , Δ <i>aliA</i> , Erm ^R	nt	this study
D39:: <i>cpsN-O</i>	D39 Δ <i>cpsN-O</i> , Δ <i>aliA</i> , Erm ^R	nt	this study
D39:: <i>cpsO</i>	D39 Δ <i>cpsO</i> , Δ <i>aliA</i> , Erm ^R	nt	this study
D39:: <i>aliA</i>	D39 <i>aliA::ermB</i> , Erm ^R	2	this study

^a Erm, Erythromycin; Cm, Chloramphenicol; ^R, resistant; ^S, sensitive; nt, non-encapsulated.

Growth curves of Streptococcus pneumoniae

Bacteria were stored at -80°C using Protect bacterial preservers (Technical Service Consultants, Heywood, United Kingdom). In order to culture the bacteria, they were streaked out on Columbia sheep blood agar (CSBA) plates and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. An overnight culture in 15 ml tubes (Sarstedt AG, St. Gallen, Switzerland) was prepared with 3 to 10 colonies in 5 ml brain heart infusion (BHI) (Becton Dickinson and Company, le Pont de Claix, France) containing 5% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). The tubes were placed in a 37°C waterbath for 9 hours without shaking. 100 μl of the overnight culture was subcultured in 5 ml BHI with 5% FCS and grown to mid-log phase ($\text{OD}_{600\text{nm}}$ 0.5-0.7). After transferring 100 μl of the mid-log phase culture into a tube with 5 ml Todd Hewitt (Becton Dickinson, le Pont de Claix, France) with 5% yeast extract (Oxoid Ltd, Basingstoke, Hampshire, England) (THY) and 5% FCS and a tube with THY alone, the $\text{OD}_{600\text{nm}}$ was measured every hour. For each strain at least three independent growth curve experiments were performed on different days. The length of the lag phase was defined as the time (minutes) needed to reach an $\text{OD}_{600\text{nm}}$ of 0.2. If a strain did not reach the $\text{OD}_{600\text{nm}}$ 0.2 in THY broth alone, a default value of 450 minutes was assigned.

Statistical analyses

Statistical analyses were done in StatView® version 5.0 (SAS Institute Inc., Cary, NC). Proportions were compared with the Chi square test or Fisher's exact test as appropriate. Differences between means were assessed by the Student's t-test. A cut-off of $p \leq 0.05$, two tailed, was used for all statistical analyses.

RESULTS

Growth characteristics of D39 capsule operon mutant

To investigate the role of capsule genes for in vitro growth, the whole capsule operon in strain D39 was knocked-out. Deletion of the capsule operon in strain D39 (strain D39::cpsA-O) led to a significantly prolonged lag phase in THY broth compared to its parent strain D39 ($p < 0.0002$) (Fig. 1). In THY broth alone, the capsule operon mutant showed a small increase of the OD with subsequent decrease during the lag phase, between 50 minutes and 120 minutes. This observation is in contrast to D39 which immediately starts to grow in THY alone. Supplementation of THY broth with 5% FCS fully compensated the prolonged lag phase of mutant D39::cpsA-O. Backtransformation of the capsule operon into D39::cpsA-O (strain D39::cpsA-O::S2) restored growth in unsupplemented THY broth (Fig. 1B).

Fig. 1A

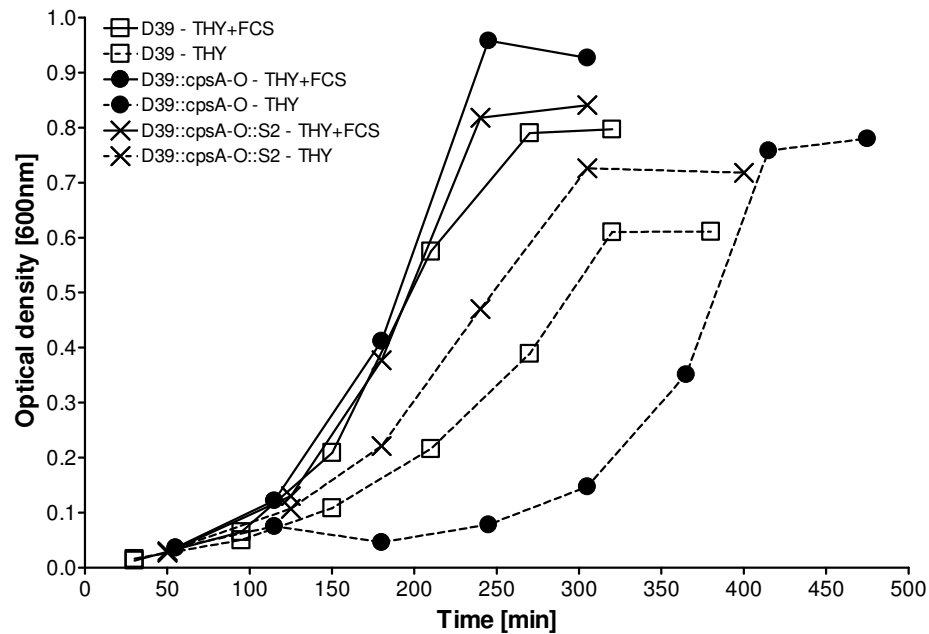


Fig. 1B

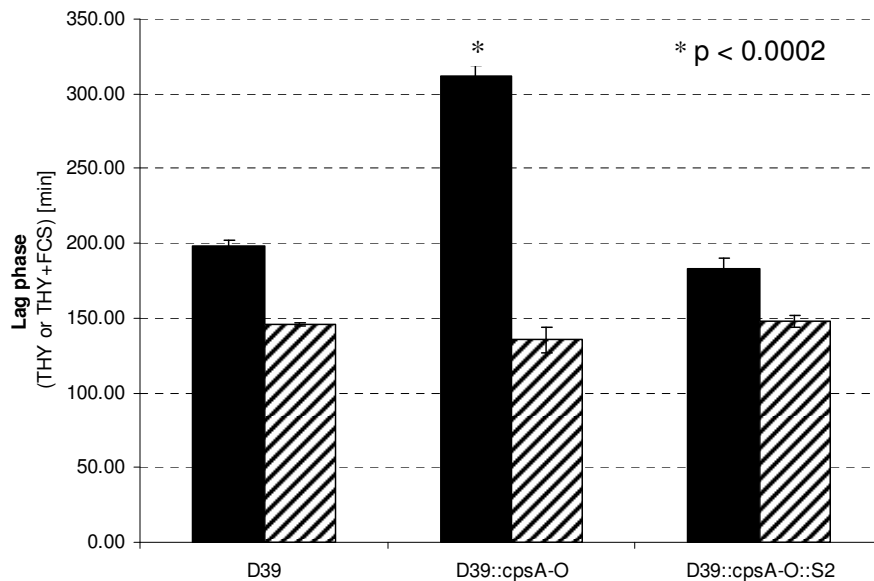


Figure 1. Growth curves **(A)** and the lag phase **(B)** in THY broth alone (broken lines, solid bars, respectively) and in THY supplemented with FCS (solid lines, striped bars, respectively) of strains D39 (serotype 2), D39::cpsA-O (non-encapsulated) and D39::cpsA-O::S2 (serotype 2). **(B)** The length of the lag phase was defined as the

time (minutes) needed to reach an OD_{600nm} of 0.2. Mean values of three independent experiments are presented (\pm SE). The p-values were calculated by comparing the duration of the lag phase of the capsule operon mutants with the duration of the lag phase of strain D39 in THY broth alone.

Growth characteristics of capsule gene deletion mutants

Mutants of D39 with partial deletion of the capsule operon showed different growth behavior (Fig. 2). Strains D39::cpsK-O and D39::cpsM-O showed a significantly prolonged lag phase in THY broth alone compared to strain D39 ($p < 0.0001$ for both mutants) (Fig. 2AB). FCS supplementation partly compensated the prolonged lag phase in mutant D39::cpsK-O and D39::cpsM-O (Fig. 2B).

To construct the capsule gene mutants, the *aliA* gene downstream of the capsule operon was deleted. To rule out the possibility that the deletion of *aliA* influenced the duration of the lag phase, the growth characteristics of an *aliA* knock-out mutant was evaluated. Knocking-out the *aliA* gene in D39 did not affect growth significantly with and without FCS supplementation compared to its parent strain D39 (data not shown).

Fig. 2A

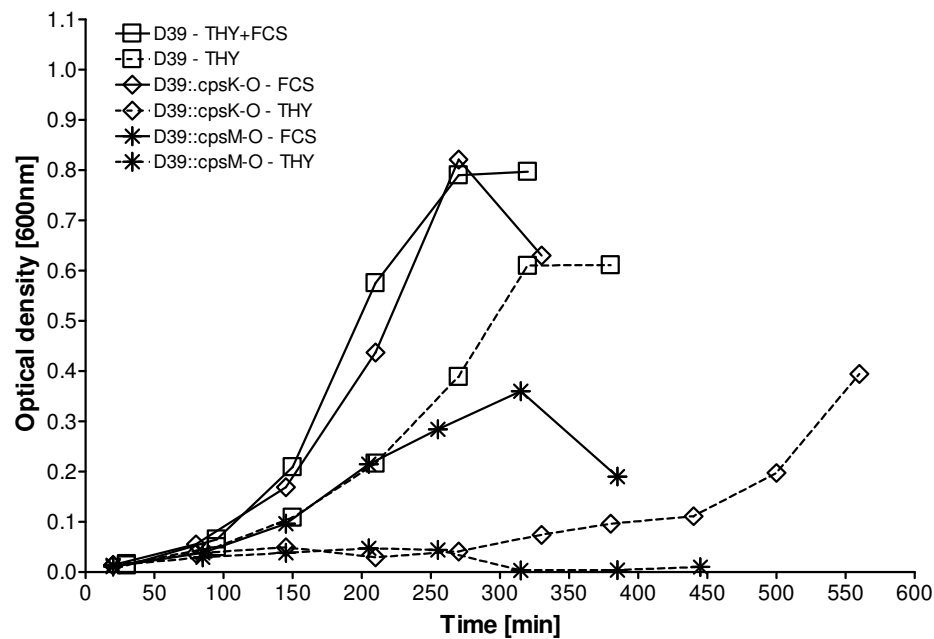


Fig. 2B

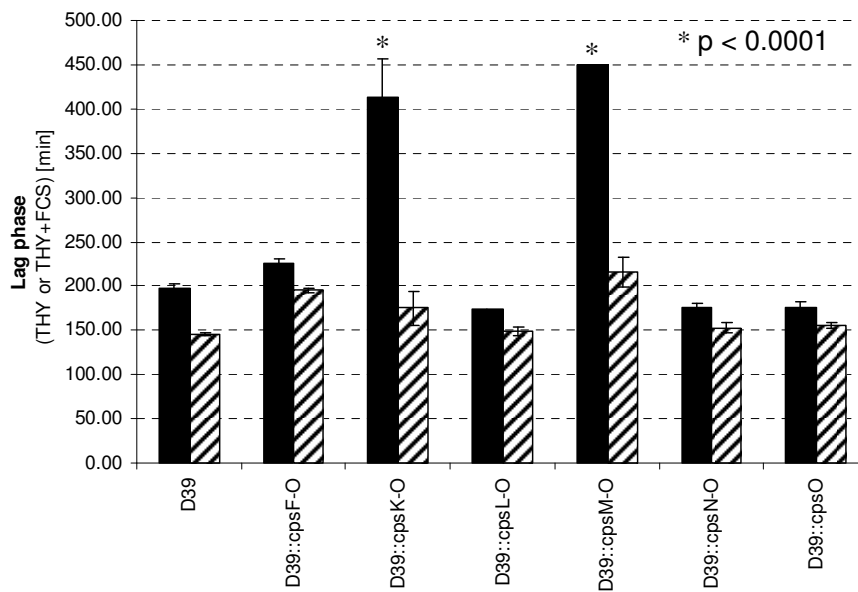


Figure 2. Growth curves **(A)** and the lag phase **(B)** in THY broth alone (broken lines, solid bars, respectively) and in THY supplemented with FCS (solid lines, striped bars, respectively) of strains D39 (serotype 2), D39::cpsF-O (non-encapsulated), D39::cpsK-O (non-encapsulated), D39::cpsL-O (non-encapsulated), D39::cpsM-O

(non-encapsulated), D39::cpsN-O (non-encapsulated) and D39::cpsO (non-encapsulated). **(B)** The length of the lag phase was defined as the time (minutes) needed to reach an OD_{600nm} of 0.2. If a strain did not reach the OD_{600nm} 0.2 in THY broth alone, a default value of 450 minutes was assigned. Mean values of three independent experiments are presented (\pm SE). The p-values were calculated by comparing the duration of the lag phase of the partial capsule mutants with the duration of the lag phase of strain D39 in THY broth alone.

DISCUSSION

Recently, we demonstrated that *S. pneumoniae* exhibits serotype-specific growth behavior [129]. Serotypes differ significantly for the duration of the lag phase during in vitro growth in unsupplemented brain heart infusion broth [129]. In addition, a prolonged lag phase was associated with enhanced invasiveness of a serotype, whereas a high colonization prevalence correlated with a short lag phase. Therefore, we hypothesized that capsule genes may have additional functions for the bacterial cell besides the synthesis of a polysaccharide capsule. This study investigated the role of the *S. pneumoniae* polysaccharide capsule genes in bacterial growth in vitro using laboratory strain D39 of serotype 2.

Knock-out of the entire capsule operon induced a prolonged lag phase in THY medium and normal growth could be restored by backtransformation of the capsule genes. This observation supports our hypothesis of a role for capsule genes in bacterial metabolism. During the lag phase the capsule gene mutant showed a slight temporary increase of the optical density before entering the phase of exponential growth. This suggests that cells started to grow initially, but were then confronted with limiting conditions. These could only be compensated for after some adaptations of the bacterium, possibly the activation of an alternative metabolic pathway.

There is some evidence for this hypothesis from observations made in other pathogens. Deletion of capsule genes in *Cryptococcus neoformans* or *Streptococcus mutans* disrupted not only capsule production, but other cellular characteristics such as growth, colony morphology, sensitivity to temperature and to nutritional environments [119-121]. Pneumococcal capsule gene products are involved in the activation and/or synthesis of carbohydrates used for capsule synthesis. Possibly, the cell also uses them for other metabolic pathways. Deletion of capsule genes may therefore induce a deficiency that must be compensated for by activation of an alternative pathway. Catabolite control protein A (CcpA) from *S. pneumoniae* has been shown to regulate the uptake of sugars [117]. Simultaneous utilization of all available sugars would be metabolically inefficient and would lead to slower growth. Therefore, CcpA suppresses genes which are involved in the uptake of nonpreferred sugars until the cell has consumed the preferred sugars or is confronted with limited conditions, e.g. due to the lack of sugar(s) resulting from capsule operon deletion.

The growth deficiency in the capsule operon mutant could be overcome by FCS supplementation. This indicates that FCS contains a rapid inducer for an alternative

pathway and/or offers a carbon source, glucogenic amino acids which can be metabolized immediately by the cell without activation of the appropriate metabolic pathway. Unfortunately, fetal calf serum is not defined. This makes it very difficult to detect the factor(s) in FCS which enhance the growth.

It has been hypothesized that the polysaccharide capsule represents stored energy, which the bacterium can use during starvation. Maybe the bacterium uses this energy store during the lag phase, when the cell is adapting to its environment. Lack of energy due to the lack of stored carbohydrates in a capsule prolong the lag phase. Partial deletions of the capsule operon had differential effects on the lag phase. Mutants lacking the serotype-specific capsule genes *cpsK-P-L-M-N-O* (D39::cpsK-O) and *cpsM-N-O* (D39::cpsM-O) showed a prolonged lag phase. Intriguingly, UDP-glucose-dehydrogenase (Ugd), which is encoded by *cpsK* in strain D39 is essential for growth at 37°C in *Cryptococcus neoformans* [120]. Along the same lines, rhamnose synthesis-deficient mutants (deletion of *cpsL-M-N-O homologues*) of *Streptococcus mutans* can only survive if they undergo additional mutations to reduce their water-insoluble glucan synthesis. [121]. Both observations point to a special role for *cpsK* and *cpsL-M-N-O* in growth and correspond with our findings.

However, mutants with deletion of capsule genes *cpsF-G-H-I-J-K-P-L-M-N-O* (D39::cpsF-O) and *cpsL-M-N-O* (D39::cpsL-O) showed normal growth. In addition, mutant D39::cpsL-O, which has two genes fewer (*cpsK* and *cpsP*) than D39::K-O, showed no growth deficiency. It is possible that enzymes encoded by capsule genes are regulated by products generated during the capsule polysaccharide production. Differential deletion of capsule genes may or may not induce, accumulation of intermediate metabolic products. The presence of a higher number of capsule genes may not necessarily be an advantage, since some intermediate products of CPS production may downregulate or inhibit other enzymes or pathway(s).

In conclusion, this study showed that pneumococcal capsule gene products influence bacterial growth in vitro and supports the observations made in *C. neoformans* and *S. mutans* [119-122]. Therefore, pneumococcal capsule genes may have multiple functions in the bacterial metabolism besides their role in polysaccharide biosynthesis. The influence of *cpsK* (Ugd) and the rhamnose genes (*cpsL-M-N-O*) on growth of strain D39 will be further investigated with single gene knock-out mutants of *cpsK*, *cpsL*, *cpsN*, *cpsM* or *cpsO*.

ACKNOWLEDGMENTS

This study was supported by a grant from the Swiss National Science Foundation to K.M., Grant No 3200-067998.

CHAPTER 7

Annex

Curriculum vitae

References

Annex

A homologue of *aliB* is found in the capsule region of nonencapsulated *S. pneumoniae*

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This study has been published in the *Journal of Bacteriology*. 2004; 186(12):3721-9.

Curriculum vitae

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Schools

1981-1987	Primary School in Worb, St. Erhard, and Nottwil, Cantons Bern and Lucerne
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1995-2001	Study of Human Medicine, Medical Faculty, University of Bern
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Languages

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Exams, courses and lectures (MD PhD)

- 2003 Molecular Microbiology exam, oral and written.
Supervisor: Prof A. Ziemiecki, Prof A. C. Andres
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- Microbiology exam, oral.
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- 2003 Practical course in Molecular Biological Methods in clinical
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- 2003 Lectures in Clinical Microbiology, University Bern
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Teaching experience

I was involved in the course preparations and the supervision of practical classes of students of biology. I introduced an undergraduate student and a PhD student into the laboratory work.

Publications

Hathaway, L.J., Stutzmann Meier, P., Bättig, P., Aebi, S., and Mühlemann, K. A homologue of *aliB* is found in the capsule region of nonencapsulated *Streptococcus pneumoniae*. **J. Bacteriol.**, 2004; 186, 3721-9.

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