Streptococcus pneumoniae:

Heteroresistance to penicillin

Roles of the polysaccharide capsule genes in growth and colonization

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PhD Thesis

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Table of contents

List of abbreviations

ABC	ATP-binding cassette
AOM	acute otitis media
ATP	adenosine triphosphate
BHI	brain heart infusion broth
BSA	bovine serum albumin
CcpA	catabolite control protein A
CCR	carbon catabolite repression
CFU	colony forming unit
CPS	capsular polysaccharides
CSBA	columbia sheep blood agar
CSP	competence stimulating peptide
DNA	deoxyribonucleic acid
dTDP	deoxythymidine disphosphate
EARSS	European Antimicrobial Resistance Surveillance System
ELISA	enzyme linked immuno sorbent assay
FBS	fetal bovine serum
FCS	fetal calf serum
GalU	glucose-1-phosphate uridylyltransferase
GalUA	galacturonic acid
Glc	glucose
GlcDH	glucose dehydrogenase
GlcNAc	*N*-acetylglucosamin
GlcUA	glucuronic acid
HIV	human immunodeficiency virus
hVISA	heteroresistant vancomycin intermediate *Staphylococcus aureus*
KanR	kanamycin resistant
KanS	kanamycin susceptible
Lac	lactose
LRPCR	long range polymerase chain reaction
Malt	maltose
MEM	minimal essential medium
MIC	minimal inhibitory concentration
MLST	multilocus sequence typing

MRSA	methicillin-resistant *Staphylococcus aureus*
NAD	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium hydrogen carbonat
OD_{600nm}	optical density at 600 nanometers
PAP	population analysis profile
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PCV7	7-valent conjugated pneumococcal polysaccharide vaccine
PFGE	pulsed-field gel electrophoresis
Pgm	Phosphoglucomutase
PTS	phosphoenolpyruvate dependent phosphotransferase system
PPV-23	23-valent pneumococcal polysaccharide vaccine
RmlA	glucose-1-phosphate thymidylyltransferase
RmlB	dTDP-D-glucose 4,6-dehydratase
RmlC	dTDP-6-deoxy-D-xylo-4-hexulose 3,5-epimerase
RmlD	dTDP-6-deoxy-L-sylo-4-hexulose reductase
rRNA	ribosomal ribonucleic acid
SCCmec	staphylococcal cassette chromosome *mec*
SE	standard eviation
SmR	streptomycin resistant
SmS	streptomycin susceptible
SOE	splicing by overlap extension
Suc	sucrose
TGase	transglycosylase
TPase	transpeptidase
THY	Todd Hewitt broth with yeast extract
TSB	tryptic soy broth
UDP	uridindisphosphate
UDP-Glc	UDP-glucose
UDP-GlcUA	UDP-glucuronic acid
UGD	UDP-glucose dehydrogenase
Und-P	undecaprenyl-phosphate
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRSA	vancomycin-resistant *Staphylococcus aureus*
VSSA	vancomycin-susceptible *Staphylococcus aureus*

Overview and summary of the thesis

Chapter 1 describes the importance of *S. pneumoniae* as a human pathogen, its epidemiology and imminent threats such as antibiotic resistance and vaccine escape mutants.

Chapter 2 introduces the two main themes of my thesis; heteroresistance to penicillin in *S. pneumoniae* and the role of the capsule operon/genes for the central metabolism. The latter divides into two parts, the influence of capsule gene *ugd* on growth and sugar metabolism, and the influence of capsule switch on growth.

Chapter 3 describes the first-time detection of heteroresistance to penicillin in *S. pneumoniae*. Heteroresistance was found in four of nine clinical pneumococcal strains and in seven of 16 international clones. Population analysis profiles of subpopulations with higher penicillin resistance showed a shift toward higher penicillin resistance levels, which reverted upon multiple passages on antibiotic free media. Conversion to homotypic resistance phenotype did, however, not occur. Comparison of two strains of the same clone revealed a correlation between the heteroresistant phenotype and a higher penicillin MIC and a greater number of altered PBP's, respectively. Future research is proposed, with the aim to find out the mechanism of heteroresistance.

Chapter 4 is divided into two parts.
First, the construction of single gene *ugd* mutant and the *ugd* complemented mutant are described. Difficulties in knocking out the *ugd* gene were encountered possible because suppressor mutants are essential for such mutants (as has been recently reported by another group). New strategies to overcome these problems are discussed.
For investigating the role of different carbon sources on growth in capsule gene/operon deficient mutants, literature was searched for chemically defined culture media. Two chemically defined media, CDM and Cden, were found, however none of the 12 tested clinical isolates reached the OD_{600nm} 0.2. Growth curves with five strains were performed in three culture media with additional components: Lacks, a semi-defined culture medium, brain heart infusion broth (BHI) and Tod Hewitt broth

with yeast extract (THY), the latter two are undefined, nutritious media. All strains grew in BHI and THY supplemented with FCS. Growth in Lacks with different supplements showed some common and some individual characteristics. Lacks media may therefore be useful to test the influence of carbon source.

Second, a total of 110 capsule switch mutants were generated.
Transformation efficacy was higher when colonizer capsule were exchanged between two colonizers strains or between two invader strains as compared to the transformation rate between a colonizer and an invader strain, and vice versa. Colonizers accepted more often capsule operons of bigger size than their original capsule operon and invader tended to prefer smaller capsule operons, especially when these capsules came from an invader.
Capsule switch mutants were analysed for their lag phase during growth in tryptic soy broth with yeast extract. Changes of the lag phase after capsule transformation were smallest when capsules were exchanged between colonizer strains and were largest when capsules were exchanged between invader strains. Invaders profited from uptake of a colonizer capsule, but colonizer strains showed a longer lag phase after transformation with invader capsules. Therefore, colonizer/invader phenotypes can be transformed between strains via capsules. This observation supports our hypothesis, that capsule genes play a role for the central metabolism.
Further experiments will characterise the capsule switch mutants and their original clinical isolates for differences in adherence in vitro to cell monolayers and colonization in an animal model, growth curves with different carbons sources, measuring cell wall integrity and gene expression by microarray assays.

Chapter 5, 6 and **7** contain references, acknowledgements and the curriculum vitae.

Chapter 8 comprises the annexes.

1. General introduction

1.1 *Streptococcus pneumoniae*

1.1.1 Microbiology

Streptococcus pneumoniae (also referred to as the pneumococcus) is a gram positive bacterium, mostly forming in diplococci, and a member of the family of lactic acid bacteria [1]. As is characteristic of the lactic acid bacteria, *S. pneumoniae* is a nutritionally fastidious facultative anaerobe requiring a complex medium for growth. This bacterium obtains energy strictly via fermentation and is incapable of respiratory metabolism, either aerobically or anaerobically [2]. Most *S. pneumoniae* strains express a polysaccharide capsule and exhibit a smooth colony morphology on blood agar plates, whereas strains without capsule show a rough colony morphology. Both types of colony are surrounded by a green zone because of α-hemolysis [3]. More than 90 serotypes have been identified based on the antigenic composition of the polysaccharide capsule [1]. Quellung reaction and Latex agglutination tests allow classification of *S. pneumoniae* into serotypes. [4].

Identification of pneumococcus isolates has traditionally relied on observations of typical colony morphology, α-hemolysis on sheep blood agar, optochin (ethylhydrocupreine hydrochloride) susceptibility and bile solubility tests [3].

One characteristic feature of pneumococci is their tendency to undergo autolysis after reaching the stationary phase and after exposure to antibiotics [5].

1.1.2 The human pathogen

As a human pathogen, S*. pneumoniae* is the most common bacterial cause of meningitis, sepsis, pneumonia, and upper respiratory tract infections, such as acute otitis media (AOM) [6], and is estimated to result in over 3 million deaths in children every year worldwide. Even more deaths occur among elderly people, among whom *S. pneumoniae* is the leading cause of community acquired pneumonia and meningitis [7]. In Switzerland, the annual incidence of reported invasive pneumococcal infections is 10/100’000 overall, 19/100’000 during the first year of life, 34/100’000 during the second year of life and 41/100’000 for people aged >64 [8]. However, true infection rates are likely to be even higher, since pneumococcal pneumonia and bacteremia often escape surveillance due to the lack of microbiological confirmation of the diagnosis.

Acute otitis media caused by *S. pneumoniae* is responsible for approximately 68'000 first outpatient visits per year in Switzerland [9]. AOM is also one of the most common reasons for antibiotic prescription in the outpatient setting in industrialized countries [10]. 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, [8, 11, 12].

The polysaccharide capsule is the most important virulence factor of *S. pneumoniae*, and nonencapsulated pneumococci are of low pathogenicity [13]. Of the overall 90 pneumococcal serotypes, 23 are responsible for >90% of all invasive infections and seven serotypes (4, 6B, 9V, 14, 18C, 19F, 23F) for about 70% of invasive infections. There has been a serotype shift during the past decades, with a decline of the previously predominant serotypes 1, 2, 3 [14]. The exact reasons for such trends are unknown, but use of antibiotics is likely a co-factor. Within the last 25 years, we have witnessed the emergence of penicillin- and multidrug-resistant *S. pneumoniae* clones, a number of which have spread worldwide [15]. Antibiotic resistance is associated more often with some distinct serotypes such as 6B, 9V, 14, 19F and 23F (the so called paediatric serotypes). Epidemic clustering of invasive pneumococcal infections has been observed especially with serotypes 1 and 5. The basis of why some serotypes exhibit a distinctive epidemiology with regard to their potential to cause invasive disease, their occurrence in specific age groups or geographic regions, their association with antibiotic resistance, and their epidemic potential, remains largely unknown [16].

1.1.3 Lifestyle

The surface of the nasopharyngeal mucosa is the major reservoir for *S. pneumoniae* and at this site it resides primarily in a commensal relationship with its human host. Carriage is extremely common, with >50% of children acquiring at least one strain during their first year of life, although an individual may harbour multiple strains simultaneously or sequentially. Each carriage episode lasts for days to months, but by age 3 carriage prevalence steadily declines until adulthood, when rates plateau at 10 to 20% [17, 18]. The capacity to occupy the nasopharyngeal niche is essential not only for invasion, but also for transmission of the organism within the human population and for genetic evolution. Progression from carriage to disease is a relatively uncommon event, but the consequences for the host are significant [19].

S. pneumoniae can mediate a range of diseases at different anatomical sites including the lung, middle ear, sinuses, blood and meninges. To survive and mediate disease in the host, the bacterium must adapt to the environments at diverse anatomical sites and adjust to differences in nutrients, osmolarity and temperature at these sites [20].

The highly populated microbial environment in the human nasopharynx provides *S. pneumoniae* with exogenous DNA from closely related oral streptococcal species and co-colonizing pneumococci. These nucleic acids can be taken up by *S. pneumoniae* because of its capacity to be naturally competent and, subsequently, be used to increase its overall fitness. The acquisition of genes that encode altered penicillin-binding proteins, for example, has facilitated resistance to ß-lactam antibiotics, which is now a common problem in the treatment of pneumococcal infections [21]. One of the most successful clones in terms of geographical dispersion and prevalence is the multidrug-resistant Spain^{23F}-1 pandemic clone, nine serotype variants of which have been identified so far. Such variants are thought to arise through natural transformation involving recombinational replacements, within and around the capsular biosynthesis locus, of DNA fragments sometimes as large as 25 kb [22, 23, 24]. The ability of the pneumococcus to take up DNA fragments and incorporate homologous sequences into its genome is only observed during aerobic growth. Indeed, this activity could also be a means of compensating for the high mutation rates that results from the oxidative lifestyle of this organism [25].

1.1.4 Vaccines

There are currently two commercially available vaccines against *S. pneumoniae*, both of which are based on the polysaccharide capsule. Pneumovax® is a 23-valent pneumococcal polysaccharide vaccine (PPV-23) which contains the most common serotypes causing >90% of invasive pneumococcal infection and is effective in adults and in immunocompetent children but not below the age of 2 years. Polysaccharides are T-cell-independent antigens and are poorly immunogenic in children under 2 years. Prevenar® is a 7-valent polysaccharide conjugate vaccine (PCV7) (containing serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) which is effective in children <2 years [26]. This vaccine can induce selective pressure and gradual replacement with non-vaccine types (serotype replacement) [27].

In the USA the incidence of invasive pneumococcal infections has been reduced by >80% since the introduction of universal vaccination with PCV7 of children in 2000

[28, 29]. Herdimmunity mediated by the reduction of nasopharyngeal colonization by vaccines serotypes 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 [10, 30, 28]. Due to the association of antibiotic resistance with pneumococcal serotypes included in the vaccine, broad administration of PCV7 has also reduced the prevalence of pneumococcal resistance [29, 31, 32, 33]. However, already during the early vaccine trials, serotype replacement by non-vaccine serotypes among colonizing pneumococci has been observed. Non-vaccine serotypes have replaced the seven vaccine serotypes among children less than 5 years old. The smallest decline was detected in serotype 19F, and the largest gain in serotype 19A. Other serotypes that more than doubled after the introduction of the vaccine were 11, 15, 33 and 35 [34, 35]. Several studies have shown an increase in carriage of serogroups 15 and 33 both in healthy children [36, 37] and in children with invasive disease [38]. One contributor to serotype replacement concerns the possibility of capsular switching, which is of great importance. The genes encoding one type of capsule are exchanged, via transformation and recombination, for the genes encoding a different type of capsule. Acquisition of a non-vaccine capsule by a pneumococcal strain capable of causing invasive disease has been a serious concern related to the use of any serotype-specific vaccine [39, 40].

1.1.5 Antibiotic resistance

During the past decades, antibiotic resistance in *S. pneumoniae* isolates has emerged and spread rapidly worldwide. Resistance has been observed to all main classes of antibiotics. Resistance rates exhibit considerable geographical variability as shown in the European Antimicrobial Resistance Surveillance Network (EARSS, www.rivm.nl/earss/) with the highest frequencies of penicillin and macrolide resistance in Spain and France. Single drug resistance is of increasing worry, but multi-drug resistance (resistance to more than two classes of antibiotics) is even more threatening and is now observed at frequencies of 10 to more than 50% in different countries [41].

Antibiotic resistance is most frequent among the prevalent paediatric pneumococcal serotypes (6B, 9V, 14, 19F, 23F). This has been ascribed to the more frequent exposure of these types to antibiotic selection pressure [42] and in turn, it has been proposed that antibiotic consumption influences serotype distribution [16]. There is

concern that antibiotic resistance may be acquired quickly by the more susceptible non-vaccine serotypes emerging under the PCV7 selection pressure.
Thus, variations in ability to colonize the nasopharynx, to accept DNA through transformation, and mutation frequencies may all be factors affecting resistance development in particular pneumococcal serotypes and not in others [43].

1.2 Heteroresistance to penicillin in *Streptococcus pneumoniae*

1.2.1 Penicillin

Penicillins belong to the β-lactam antibiotics, which are important in the treatment of gram-negative and gram-positive infections due to *E. coli, Klebsiella pneumoniae, Neisseria meningitidis, Hämophilus influenzae, Streptococcus pneumoniae*, staphylococci and enterococci. These agents represent >65% of the world antibiotic market with >50 marketed drugs of this class. Beside penicillins, β-lactams include cephalosporines, carbapenems, monobactams [44, 45] and more recently the penicillin-cephalosporin hybrids, the penems (e.g. faropenem) [46]. Characterized by a four-membered β-lactam ring (Fig. 1), these agents target the bacterial enzymes of cell wall biosynthesis, the so-called penicillin-binding proteins (PBP's), although the actual mechanism of killing is as yet unresolved [47]. Resistance to this agents occurs as a result of drug inactivation by β-lactamases, target site (i.e. PBP) alterations, diminished permeability and efflux [44].
Pneumococcal resistance to penicillin has spread tremendously over the world since the first clinical isolate resistant to penicillin was described in 1967, where it was recovered from a patient in Papua New Guinea [48]. The sentinel event in the epidemiology of antibiotic-resistant pneumococci was the outbreak in 1977 in South Africa of pneumococcal diseases caused by multidrug-resistant strains [49]. Today more than 50% of isolates in some areas may carry this resistance trait. In the United States a dramatic increase has been noticed of penicillin resistant pneumocooci from 3 – 5% during 1980s to 34% in 1999 – 2000 [50]. In Switzerland, overall penicillin resistance rates have remained relatively low (10%, of which 2 % are high-resistant) [8, 11]. 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%) [8, 11, 16, 51].

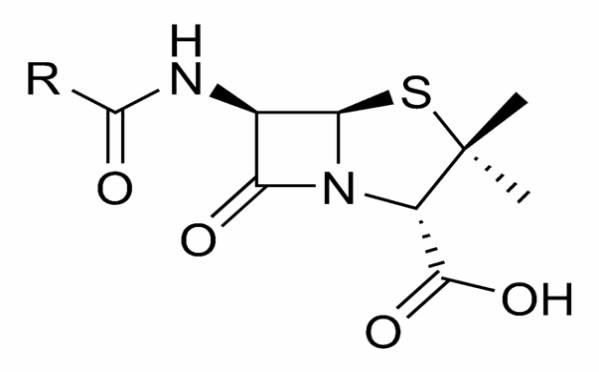

Figure 1. Core structure of penicillins (www.wikimedia.org/82/Penicillin-core.png)

1.2.2 Resistance mechanisms against β-lactam antibiotics

The mechanism of penicillin resistance in clinical isolates of *Streptococcus pneumoniae* involves the production of penicillin binding proteins (PBP's) with reduced affinitiy for the β-lactam antibiotic molecule [52, 53, 54]. This reduced affinity is a result of heterologous recombinational events and spontaneous point mutations that lead to alterations in the genetic determinants of these proteins. The resistant genes probably have evolved from sensitive closely related oral streptococci that contain genes closely related to the sequences found in the mosaic blocks of resistant *S. pneumoniae pbp* genes [55]. According to their overall domain structure, PBP's are classified as high molecular weight PBP's of class A possessing an N-terminal glycosyltransferase domain, high molecular weight PBP's of class B with an N-terminal domain of unknown function, and low molecular weight PBP's that act mainly as D,D-carboxypeptidases [56]. Thus, *S. pneumoniae* contains six PBP's, named PBP 1a, 1b, 2x, 2a, 2b and 3. [55, 57, 58]. Although resistant (low β-lactam affinity) forms are known for all 6 PBPs, modification in PBP 1a, 2b and 2x are required for high resistance to penicillin to develop [59].

Since the capsular gene locus is flanked by the *pbp1a* and *pbp2x* genes, intraspecies transformation of resistance can result in capsular switching which has has been shown to occur in natural populations. The recombination event in this case involves one or both PBP-encoding genes, as well as the capsule locus, and leads to a change of both the serotype and the resistance profile of the strain [60]. Trzcinski et al. could show that resistance was associated with a fitness cost immediately after gene transfer in 3 different sets of pneumococcal strains (isogenic variants differing only in their resistance to β-lactams). The cost increased with the degree of resistance conferred by additional *pbp* gene substitutions [61]. PBP's are biosynthetic enzymes with transpeptidase (TPase) and/or transglycosylase (TGase) activity that

catalyse terminal stages in the synthesis of bacterial peptidoglycan [58] and it has been proposed that the reduced reactivity of these proteins for the antibiotic molecule in resistant mutants may also bring along changes in their catalytic efficiency with respect to their physiological substrates [62, 63].

Nevertheless, the increased minimal inhibitory concentration (MIC) value of such penicillin-resistant isolates is not simply a consequence of the reduced reactivity of the antibiotic targets, as inactivation of genes other than the *pbp* determinants can also influence resistance levels significantly. A virtually complete loss of resistance was observed in penicillin-resistant mutants in which the *murMN* operon was inactivated [64]. But not all penicillin resistant clinical isolates contain altered murein and altered *murMN* alleles. The MurM protein catalyses the first step in the addition of short dipeptide branches to the muropeptide units of the pneumococcal peptidoglycan [64, 65]. Similarly to the case of *murM* mutants, inactivation of the pneumococcal muramic acid O-acetylase (*adr*) produced three phenotypes: changes in the covalent structure of the muropeptide components of peptidoglycan (elimination of O-acetyl groups), hypersensitivity to lysozyme and reduction in the penicillin MIC value [66]. O-acetylation was shown to protect *S. aureus* against exogenous lysozymes [67]. Further the *cpoA* gene encoding a putative glycosyltransferase which is involved in cell wall biosynthesis seems to play a role in pneumococcal resistance. Mutants in *cpoA* were obtained by selection with the lytic β-lactam piperacillin but not with cefotaxime. Probably *cpoA* relates to the trigger mechanisms of the major autolysin, that is responsible for β-lactam induced lysis, a process which is still not understood at all [53].

Also the CiaRH two component system seems to play a role in pneumococcal resistance to β-lactams. Cefotaxime resistant laboratory mutants contained mutations in the histidine protein kinase CiaH, with every mutant containing a different *ciaH* allele [68]. The CiaRH two component system is required during cell wall stress: deletion mutants in *ciaR* are unusually lysis prone and hypersensitive to a wide variety of early and late cell wall inhibitors like β-lactams, vancomycin, bacitracin, D-cycloserine, whereas mutants with an activated CiaRH system were highly resistant to many different lysis inducing conditions. Moreover, deletion of the response regulator in mutants containing a low affinity PBP2x showed severe growth defects and lysed rapidly. This strongly suggests that PBP2x mutations are functionally not neutral, and that this defect can be balanced by a functional CiaRH system [69].

In summary, the evolution of β-lactam resistance in *S. pneumoniae* represents a highly complicated scenario, involving target proteins such as PBP's and other regulatory components.

1.2.3 Heteroresistance

1.2.3.1 Definition

The term "heteroresistance" has not yet been clearly defined, but it is usually understood as the presence of one or several bacterial subpopulations at a frequency of 10^{-7} to 10^{-3}, which can grow at higher antibiotic concentrations than predicted by the minimal inhibitory concentration (MIC) for the majority of cells [70]. Heteroresistance to penicillin has not yet been described in *Streptococcus pneumoniae.*

Recently Wolter et al. reported heterogeneous macrolide resistance in *Streptococcus pneumoniae*. A macrolide resistant clinical isolate of *Streptococcus pneumoniae* with 23S rRNA mutations showed a heterogeneous phenotype and genotype. In the Etest for erythromycin a zone of inhibition was observed as is typical of a susceptible strain, however satellite colonies occurred at a higher MIC [71]. Because the resistance mutations occur in genes present in multiple copies, such as the 23S rRNA genes in macrolide resistance [72], the study of resistance mechanisms is complicated by gene conversion. Culture of a resistant strain in the absence of antibiotic pressure showed gene conversion to occur between the four 23S rRNA alleles, resulting in reversion to susceptibility. Reduced growth rate in the resistant strain suggests that the mutations were associated with a fitness cost [71]. Most reports about heteroresistance are about methicillin-resistant *Stapylococcus aureus* (MRSA) and vancomycin-intermediate resistant *Staphylococcus aureus* (VISA).

1.2.3.2 Heteroresistance in *Staphylococcus aureus*

Methicillin resistance in *Staphylococcus aureus* is due to the acquisition of a large DNA element, termed staphylococcal cassette chromosome *mec* (SCC*mec*) [73]. The prerequisite for methicillin resistance located on SCC*mec* is *mecA*, which encodes a low-affinity penicillin-binding protein PBP2a. A characteristic of methicillin resistance is its usually heterogeneous expression, which means, that growth in the presence of β-lactams selects highly resistant subclones from an MRSA population with low methicillin MIC's. The frequency at which highly resistant subclones arise is

a reproducible, strain-specifc characteristic [74] and usually lies clearly above the rate of spontaneous mutation, but is not likely a mutator phenotype [70]. With few exceptions [75], once high level resistance has been selected, it remains high. In contrast to the homogeneous resistance phenotype, heteroresistant strains show different colony sizes [70].

MRSA strains could be divided into four expression classes based on the shape of the PAP (population analysis profile) curves [74]. The great majority of cells (99 %) of class 1 strains showed MIC's not much greater than those for susceptible staphylococci. The PAP's of most class 2 strains showed the presence of several subpopulations with MIC's intermediate between those of the majority of cells and those of the most highly resistant bacteria. In class 3, strains had high resistance levels plus usually a single subpopulation of very highly resistant cells. Class 4 cultures were composed of cells of uniform and very high methicillin resistance. To elucidate the causes of heteroresistance, a number of genetic changes were characterized whose activity affects the level of resistance [76]. Many of these genes are involved in cell wall biosynthesis and their study has given valuable insight into this pathway. However, none of these so-called *fem* or *aux* factors has been shown to be the central effector of heteroresistance in *Staphylococcus aureus* [77].

Heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) has been defined as strains that are susceptible to vancomycin, (MIC ≤4mg/L) [78] but contain a subpopulation of cell at a frequency of ≥ 10^{-6} that exhibit intermediate susceptibility (MIC >4mg/L but <32mg/L). Vancomycin is a glycopeptide antibiotic that has as its primary target the D-ala-D-ala subunits of the gram-positive cell wall, which causes cell death by inhibiting cell wall cross-linking [79].

Vancomycin-intermediate *Staphylococcus aureus* (VISA), first described in 1997 [80], has continuously been a worldwide problem in the treatment of methicillin-resistant *S. aureus* hospital infections [81]. VISA has a unique mechanism of resistance; the resistant cell produces a thickened cell wall, whereby many vancomycin molecules are trapped within the cell wall. The trapped molecules clog the peptidoglycan meshwork and finally form a physical barrier towards further incoming vancomycin molecules [82, 83, 84]. VISA does not directly emerge from vancomycin-susceptible MRSA. It emerges from hetero-VISA that expresses heterogeneous-type vancomycin resistance [80].

Compared to vancomycin resistant *S. aureus* (VRSA), where a simple intake of the genetic system (*vanA, vanH, vanY*, and *vanX*) [85] completes the drastic alteration of

cell wall peptidoglycan composition, the acquisition of vancomycin resistance by VISA is based on spontaneous mutations [86] and on mobile genetic elements, like insertion elements in the *yycFG* promoter region [87]. Neoh et al could show that a mutated response regulator *graR* is responsible for phenotypic conversion of *Staphylococcus aureus* from heterogeneous vancomycin-intermediate resistance to homogeneous vancomycin-intermediate resistance. The physiological function of *graR* is to regulate the transcription level of two ABC transporter genes. The expression of the mutated *graR* in hVISA caused remarkable cell wall thickening and an increase in the vancomycin MIC to the level seen for VISA strains. However, overexpression of *graR* in vancomycin-susceptible strains did not cause that significant effect [86].

Sakoulas et al. investigated the possibility of cross-heteroresistance between vancomycin and daptomycin in methicillin-resistant *Staphylococcus aureus*. In three of four patient series, treatment with vancomycin was associated with the development of vancomycin heterogeneous resistance and accompanied by daptomycin heteroresistance [88]. The expression of *graR* in hVISA increased not only vancomycin resistance but also daptomycin resistance [86].

1.2.3.3 Heteroresistance in other pathogens

Resistance heterogeneity is not confined to *Staphylococcus aureus* but is also described for other pathogens.

Coagulase-negative-staphylococci: including *S. epidermidis*, *S. capitis, S. haemolyticus, S. auricularis, S. simulans and S. warneri* have also shown a heteroresistance to methicillin or vancomycin [89, 90].

Acinetobacter baumannii: heteroresistance to colistin in multridrug-resistant *Acinetobacter baumannii* clinical isolates was reported that were susceptible to colistin based on MIC's. Case histories showed that the patients from whom the isolates were obtained had not been exposed previously to colistin. The proportion of resistant subpopulations was increased after exposure to colistin. Substantially decreased proportion of the colistin-resistant subpopulations after passage in drug-free broth has shown that they were not stable mutators. The detection of heteroresistant *A. baumanni* to colistin in clinical isolates provides a warning because colistin is often the last-line antibiotic and its inappropriate use may have substantial potential for the rapid development of resistance and therapeutic failure [91].

Enterococcus faecium: Heteroresistance to vancomycin was discovered in a clinical isolate of a patient with endocarditis. Although the clinical isolate was susceptible in vitro when tested by microtiter broth dilution, follow-up E-tests showed subcolonies present in the clear zone of inhibition. After eight serial passages in vancomycin containing broth, the susceptible isolate became resistant. Both the susceptible and resistant colonies were from the same strain as determined by pulsed-field gel electrophoresis (PFGE), and both contained the *vanA* gene as determined by PCR [92].

Cryptococcus neoformans: Mondon et al. investigated serial isolates of *Cryptococcus neoformans* from two infected patients and demonstrated that each isolate produced cultures with heterogeneous compositions of fluconazole susceptibility and that the proportion of subpopulations resistant to fluconazole increased steadily over time. Highly resistant clones were observed in these isolates at frequencies ranging from 0.7 to 4.6 %. Homogeneous, highly resistant clones were selectable on medium with a high drug concentration after a single passage. However high level resistance was reversible after serial passage in drug-free medium [93]. Xu et al concluded that the mutation leading to fluconazole resistance in *C. neoformans* is a dynamic and heterogeneous process and speculated that multiple mechanisms for acquisition of drug resistance exist in this species. Interestingly, the MIC's for the mutants developed by Xu et al were stable after subculture in drug-free medium [94].

Candida albicans: An instable resistance was also seen among heteroresistant isolates of *Candida albicans*. Marr et al identified heterogeneous compositions in fluconazole susceptibility in serial isolates of *C. albicans* from two bone marrow transplant patients. Rapid induction of highly resistant isolates, which subsequently caused disseminated infection, during fluconazole therapy was found to be associated with this heterogeneous phenotype [95].

1.3. Roles of *Streptococcus pneumoniae* polysaccharide capsule genes in growth

1.3.1. The polysaccharide capsule

The polysaccharide capsule forms the outermost layer of *S. pneumoniae* cells, and is approximately 200 – 400 nm thick [96]. During infection it inhibits phagocytosis by polymorphonuclear leukocytes [97].

Factor (typing) sera are used to divide pneumococci into serotypes and serogroups, which include immunologically related serotypes. At present, 91 individual serotypes are recognised by their patterns of reactivity with the factor sera [98]. Each serotype has a structurally distinct capsular polysaccharide (CPS) composed of repeating oligosaccharide units joined by glycosidic linkages. The simplest CPS types are linear polymer with repeat units comprising two or more monosaccharides. The more complicated structural types are branched polysaccharides with repeat unit backbones composed of one to six monosaccharides plus additional side chains [99]. The capsular polysaccharide of some serotypes (2, 4, 6A, 6B, 7F, 8, 14, 19F, and 23F) have shown to be covalently linked to the cell wall peptidoglycan [100].

Serotypes vary in the extent to which they are carried in the nasopharynx and the degree to which they are recovered from different disease states. Typical “invasive” serotypes are 1, 4, 5, 7F, 9V, and 14 and “colonizing” serotypes are 3, 6A, 6B, 18C, 19F and 23F. By definition, invader, describes a group of serotypes with low colonization prevalence but relative high frequency of invasive disease [39, 101, 102]. The underlying mechanism that renders some serotype more invasive or colonizing than others is not understood.

Expression of a capsule is important for survival in the blood and is strongly associated with the ability of pneumococci to cause invasive disease. Antibodies against CPS provide protection against pneumococcal disease. The substantial diversity of pneumococcal CPS’s is believed to have arisen as a consequence of selection for antigenic diversity imposed by the human immune system [98]. The amount of capsule produced depends on phase variation, oxygen tension and close contact with an epithelial surface [103, 104, 105].

The most frequently found carbohydrates in pneumococcal capsular polysaccharides are α/β-D-glucose, α/β-D-galactose, α/β-L-rhamnose, *N*-acetyl-α/β-D-glucosamine, *N*-acetyl-α/β-D-galactosamine, *N*-acetyl-α/β-D-mannosamine, *N*-acetyl-α-L-fucosamine, α/β-D-Glucuronic acid. Also present may be α–L-fucose (serotype 19A),

β-D-ribose (serotype 7B, 19B, 19C), α-D-galacturonic acid (serotype1), *N*-acetyl-β-D-mannosaminuronic acid (serotype 12A, 12F), *N*-acetyl-α-L-pneumosamine (serotype 5), 2-acetamido-4-amino-2,4,6-trideoxy-α-D-galactose (serotype 1), 2-acetamido-2,6-dideoxy- α-D-xylo-hexos-4-ulose (serotype 5) [106].

1.3.2 The capsule operon

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) [107, 108]. The first four genes of the *cps* locus (*cpsA-D*) are common to all pneumococcal serotypes, with the exception of serotypes 3 and 37. The proteins, that are encoded by these genes, *cpsA-D*, are involved in regulation and export of CPS. [109]. The central region of the *cps* locus comprises genes that encode specific glycosyltransferases that assemble the serotype-specific oligosaccharide repeat unit on a lipid carrier. This region also includes a flippase (Wzx) that transports the repeat unit to the external face of the membrane and a polymerase (Wzy) that links the units together.

The final region of the locus comprises genes that encode the synthesis of activated sugar precursors, some of which are common to several CPS types, e.g. those encoding dTDP-L-rhamnose or UDP-glucuronic acid synthesis. [25, 100]. Almost all capsule loci comprise up to 20 or more genes [111], that forms a single transcriptional unit [7].

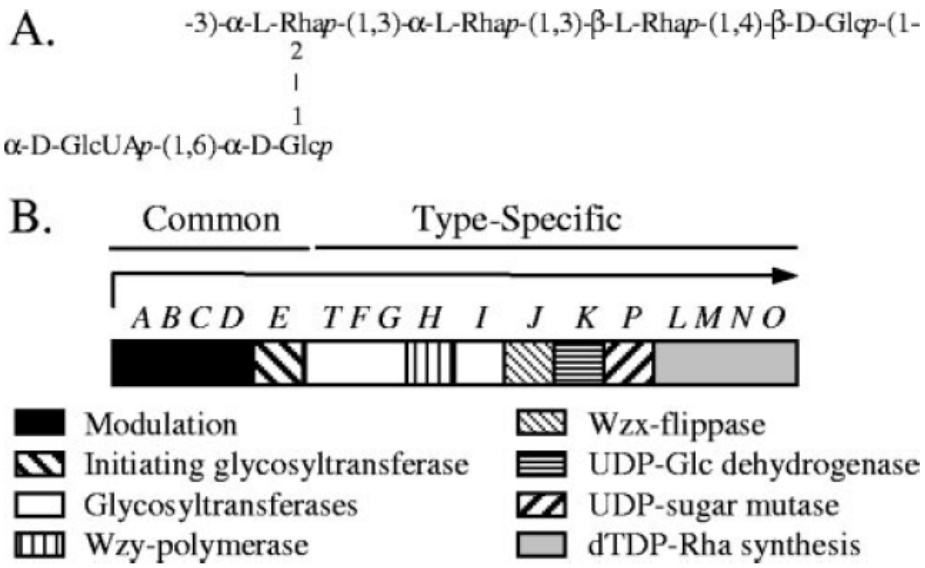

Figure 2. Type 2 capsule polysaccharide structure and genetic locus. A) Structure of the repeat unit. Synthesis of the backbone initiates by addition of Glc-1-P to a polyprenol acceptor. B) Genetic organization of the type 2 capsule locus. The arrow indicates the putative transcript containing *cps2A* to *cps2O* [112].

1.3.3 The biosynthesis of the polysaccharide capsule

With the exception of types 3 and 37 which are synthesized by the synthase pathway [113, 114] pneumococcal CPS's are generally synthesised by the Wzx/Wzy-dependent pathway (Fig. 3) [98]. CPS's are synthesized by transfer of an initial monosaccharide phosphate from a nucleotide diphosphate sugar to a membrane-associated lipid carrier, followed by the sequential transfer of further monosaccharides to produce the lipid-linked repeat unit. This is transferred to the outer face of the cytoplasmic membrane by the repeat-unit transporter or flippase, polymerized to form the mature CPS, and then attached to the peptidoglycan [99].

Most of the carbohydrates required for polysaccharide synthesis are synthesized by enzymes encoded by the capsule operon, but some precursors that also occur in other cellular structures, such as glucose, *N*-acetylglucosamine (GlcNAc) (of peptidoglycan), and ribitol (of teichoic acids), are synthesised by enzymes encoded by genes located outside the capsule operon [108, 115, 116].

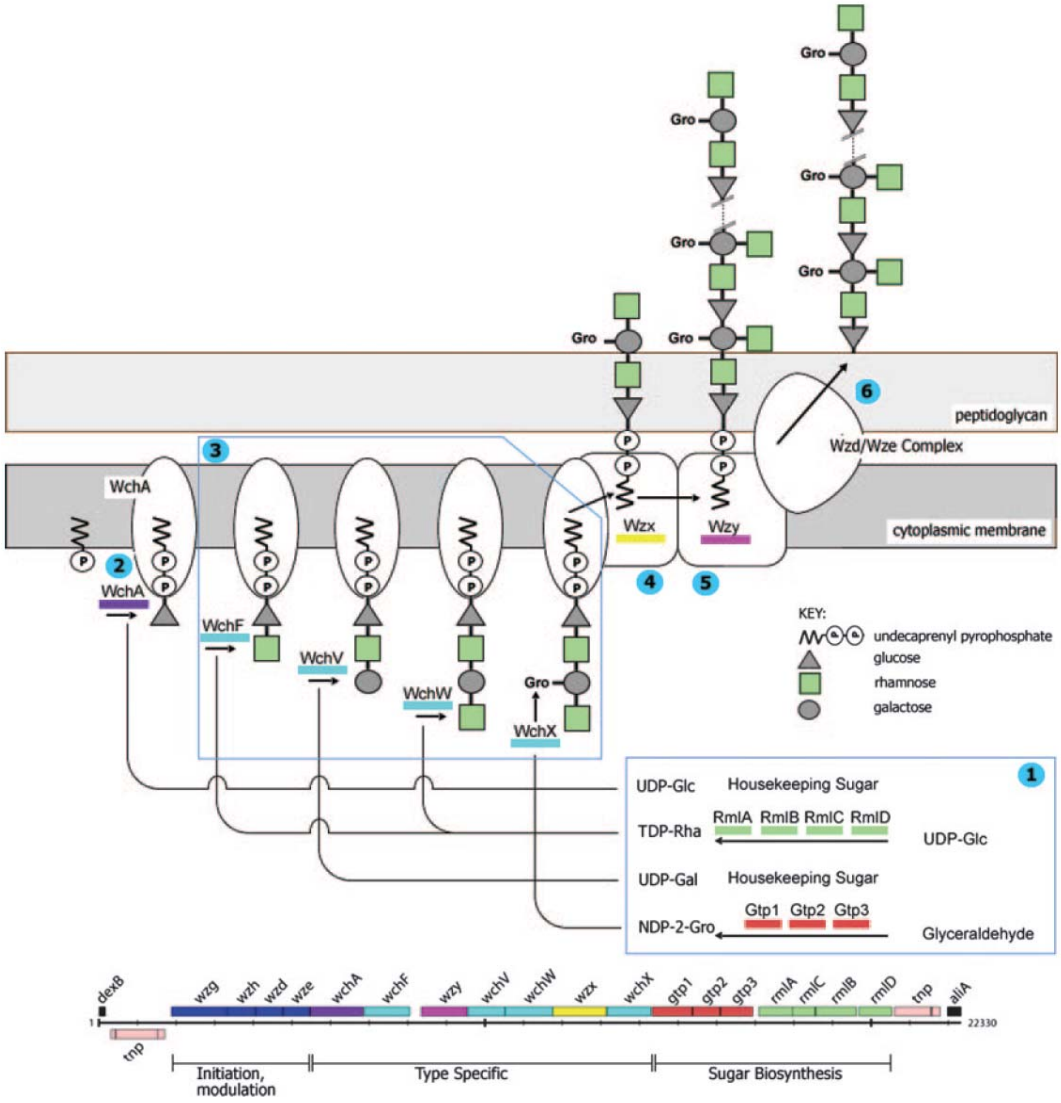

Figure 3. Schematic representation of the biosynthesis of CPS by the Wzy-dependent pathway. The biosynthesis of the CPS of serotype 23F is represented [117].

1) UDP-linked components of the repeat CPS unit are synthesized by genes encoded within the *cps* locus or are available from central metabolism
2) Repeat unit biosynthesis is initiated by the transfer of glucose phosphate to the lipid carrier by the initial transferase WchA
3) Sequential addition of the other components of the repeat unit catalyzed by the glycosyltransferases
4) The lipid-linked repeat unit is transferred across the membrane by the Wzx flippase
5) Wzy polymerase links individual repeat units to form lipid-linked CPS
6) The lipid-linked CPS is linked to the cell wall by a poorly understood process involving the Wzd/Wze complex, with release of the undecaprenyl phosphate carrier

1.3.4 Regulation of capsular polysaccharide production

CPS biosynthesis of all but two pneumococcal serotypes has been shown to be dependent upon a regulatory system that is determined by CpsB, CpsC and CpsD Fig. 4). CpsB is a manganese-dependent phosphotyrosine-protein phosphatase, CpsC is a membrane protein that is related to polysaccharide co-polymerases and

CpsD is an autophosphorylating protein-tyrosine kinase. CpsC is required for CpsD tyrosine autophosphorylation; in a *cpsC*-deletion mutant, CpsD does not become phosphorylated. Mutation of the *cpsD* gene to inactivate the ATP-binding site eliminated CPS production [118]. CpsB is required to dephosphorylate CpsD; in *cpsB*-deletion mutants, the proportion of CpsD that is phosphorylated increases dramatically, and there is a significant decrease in the amount of CPS that is produced. Therefore the non-phosphorylated form of CpsD is active in CPS biosynthesis.

Recently, a novel role for CpsC in the attachement of CPS to the pneumococcal cell wall was also identified [100]. Thus, CpsB, CpsC and CpsD function together to regulate CPS assembly, export and attachement to the cell wall by tyrosine phosphorylation of CpsD [100, 118, 119]. Therefore, modulation of capsule chain length and amount occurs, at least in part, through the action of these autophosphoregulatory system that includes an autophosphorylating tyrosine kinase. CpsC and CpsD represent the membrane-associated activation domain and cytoplasm-associated ATPase domain, respectively of this kinase (Fig. 4) [112].

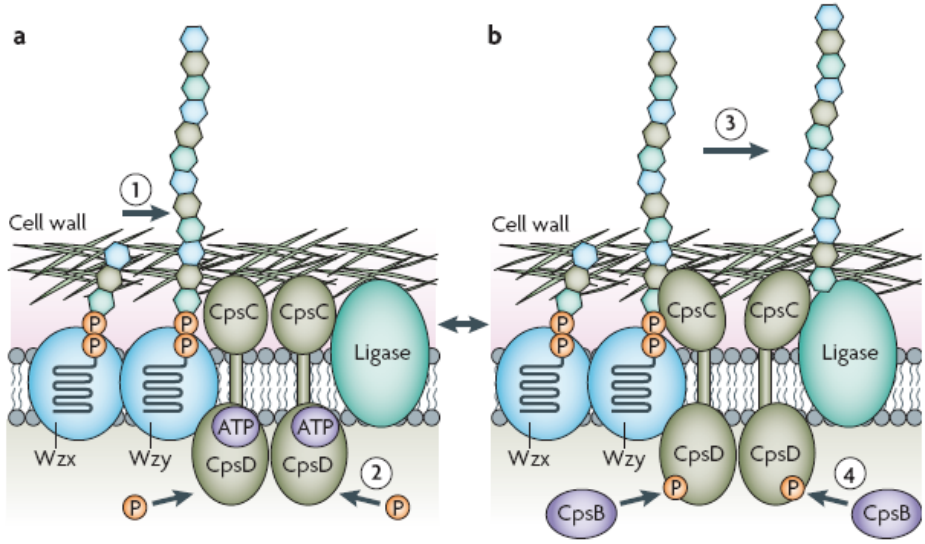

Figure 4. Model showing the regulation of capsular polysaccharide (CPS) production by tyrosine phosphorylation of CpsD. a) CpsC, CpsD and ATP interact to promote CPS biosynthesis by the polysaccharide polymerase (step1). CpsD autophosphorylates, which causes a change in protein interactions and slows CPS biosynthesis (step2). b) The CPS polymer is then transferred to the putative CPS cell-wall ligase, and is ligated to the cell wall (step3). Finally, CpsB dephosphorylates CpsD, thereby allowing the cycle to be repeated (step4) [25].

1.3.5 Role of the polysaccharide capsule in colonization

Immediately after entering the nasal cavity, *S. pneumoniae* cells encounter mucus secretions. The expression of a capsule reduces entrapment in the mucus, thereby allowing the pneumococcus to access the epithelial surfaces [120]. Almost all pneumococcal CPS's are negatively charged, which could increase their repulsion from the sialic acid-rich mucopolysaccharides that are found in mucus [106]. This might allow bacterial access to receptors on the apical surface of the epithelial cells that line the nasal spaces. Once at the epithelial surface, the expression of a thick capsule seems to be disadvantageous for the pneumococcus, because of its inhibitory effect on adherence [120].

Most pneumococcal isolates that have been investigated display phase variation between two forms that can be distinguished by their opaque or transparent colony morphologies. During the initial stages of colonization, transparent variants that express a thinner capsule and possess other characteristic that promote binding to host receptors prevail over opaque variants [104]. In both mouse models and in humans, opaque variants that express increased amounts of CPS and are more resistant to opsonophagocytic killing are selected for during the transition from the mucosal surface to the bloodstream [121, 125]. The differences in virulence between pneumococcal serotypes are a function of the biological properties of the CPS itself and is not simply related to the thickness of capsule [122].

1.3.6 Sugar metabolism in *Streptococcus pneumoniae*

S. pneumoniae depends on external sugars for its energy requirement. Compared to human blood (3.57-6.06 mM), glucose levels at other common sites of streptococcal infection are generally quite low (saliva 0.02-0.4 mM, nasal secretion <1.0 mM, lower airway secretions <0.5mM), meaning that alternative energy sources need to be pursued [123].

Over 30% of the transporters in *S. pneumoniae* are predicted to be sugar transporters, which is the highest percentage observed to date in any sequenced prokaryote [124]. These transporters include classical PTS (phosphoenolpyruvate (PEP)-dependent phosphotransferase system), ATP-binding cassette (ABC) and ion gradient-driven transporters. *S. pneumoniae* has 21 PTS sugar-specific enzyme II complexes with a variety of genes and domain arrangements [7]. The *S. pneumoniae* PTS includes systems specific for fructose, glucose, lactose, mannose, mannitol, trehalose, *N*-acetylglucosamine, and sucrose, as well as a variety of PTS systems

whose sugar specificities remain to be determined. The ability of *S. pneumoniae* to metabolize this wide range of sugars may confer a fitness boost in certain host niches [7]. Furthermore, *S. pneumoniae* produces three surface-associated exoglycosidases: a neuraminidase, NanA, a β-galactosidase, BgaA, and a β-*N*-acetylglucosaminidase, StrH. These enzymes act sequentially to remove the terminal sugars that are found on many human glycoconjugates and, therefore, might unmask receptors for adherence, thereby affecting the function of glycosylated host clearance molecules and/or providing a nutrient source [125]. Therefore, host glycoproteins and murein polysaccharides, as well as its own capsular polysaccharides, may be major sources of sugars for *S. pneumoniae* and thereby contributing to its effective growth and colonization of the nasopharynx. [7].

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 [126]. But the microorganism can also grow on many other sugars, when provided as the only carbon source. The ability to use preferred sugars depends on a regulatory process called carbon catabolite repression (CCR). CCR causes silencing of genes specific for the utilization of nonpreferred sugars until the cell has consumed the preferred sugar(s) [126]. The catabolite control protein (CcpA) is the main global regulator of CCR in *Bacillus subtilis* [127] and regulates catabolite operons also in many Streptococcus spp. including *Streptococcus mutans* (where it has been shown to be required for biofilm formation) [128]. CcpA belongs to the LAcI/GalR family of activator-repressor transcription factors [126]. CcpA binds to catabolite–responsive elements (*cre*) located within or near promoters. If the *cre* is located within the promoter region or open reading frame, binding of CcpA inhibits RNA polymerase interaction with the promoter or its progression through the DNA, thereby repressing transcription [129]. Binding of CcpA to a *cre* located upstream of the promoter is proposed to enhance transcription by allowing CcpA to interact with RNA polymerase [130]. CcpA binding to *cre* is enhanced by the binding of Ser46-phosphorylated HPr to CcpA. HPr is a component of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), which is also regulated by CcpA [131].

In *S. pneumoniae* CcpA (also called RegM) regulates both β-galactosidase and α-glucosidase but is not involved in glucose repression of either of these enzymes. Glucose repression of β-galactosidase was partially mediated via CcpA, however a secondary unknown regulator was also involved [132]. Inactivation of CcpA in *S. pneumoniae* resulted in decreased virulence in a mouse model of bacteremia.

Polysaccharide capsule gene expression was decreased in the *RegM* mutant thereby providing a possible explanation for the effect of RegM inactivation on pathogenesis [133].

The role of sucrose utilization in *S. pneumoniae* virulence recently was demonstrated. Iyer et al. discovered that a sucrose ATP-binding-cassette (ABC) transport system contributed to the ability of S*. pneumoniae* to cause pneumonia, whereas a sucrose PTS was important for colonization of the nasopharynx. Both sucrose uptake systems are members of the LacI family [123].

This role for a carbon regulator in the control of genes associated with metabolism and in vivo fitness connects the physiological process of carbon and sugar metabolism with colonization and disease [126].

1.3.7 Biosynthesis and role of glucuronic acid

UDP-glucuronic acid (UDP-GlcUA) is used by many pathogenic bacteria in the construction of an antiphagocytotic capsule that is required for virulence. In mammalian polymers such as hyaluronan, chondroitin sulfate and heparin sulfate, glucuronic acid is part of the backbone structure and is also utilized in the process of hepatic glucuronidation of potential toxins [112, 134]. Depending on the cell type, glucuronic acid may be further converted to UDP-xylose, UDP-arabinose or UDP-galacturonic acid and therefore could potentially contribute to important modifications of proteins, lipids, or other glyconconjugates [135]. The enzyme UDP-glucose dehydrogenase (UDP-GlcDH) catalyzes the NAD+-dependent twofold oxidation of UDP-glucose (UDP-Glc) and provides a source of glucuronic acid (Fig. 5). UDP-glucose-dehydrogenase is encoded by the *ugd* gene (*cps2K* in D39) and is located in the serotype specific region of the capsule operon [112] in the following capsule loci: 1, 2, 3, 5, 8, 9A, 9L, 9V, 17A, 22A, 22F, 25F, 31, 38, 41A, 41F, and 45. The capsule of serotype 1 and 25F contain galacturonic acid (GalUA) and not GlcUA [106]. In serotype 3 UDP-glucuronic acid levels correlate with capsule production. A reduction in cellular UDP-GlcUA levels leads to diminished type 3 capsule production by reduction of the lengths of the polysaccharide chains [136]. Overexpression of UDP-GlcDH in *E. coli* resulted in decreased production of the K5 capsule (which consists of GlcUA-GlcNAc) but the polymer chain lengths were unaltered, suggesting a reduction in the number of chains. A consequence of an increase in the levels of UDP-GlcUA within the cell may be that excess UDP-GlcUA is able to transiently

occupy the binding site for UDP-GlcNAc and thus function as a competitive inhibitor and thereby lower the K5 production [137].

The eukaryotic enzyme UDP-glucose dehydrogenase is a homohexamer of 52 kDa subunits, whereas the bacterial UDP-GlcDH is reported to be active as a homodimer of 47 kDa subunits in *E. coli* and as a monomer in Group A streptococci. The conserved primary sequences of the prokaryotic and eukaryotic UDP-GlcDH enzymes suggest that they are likely to use a common catalytic mechanism [137].

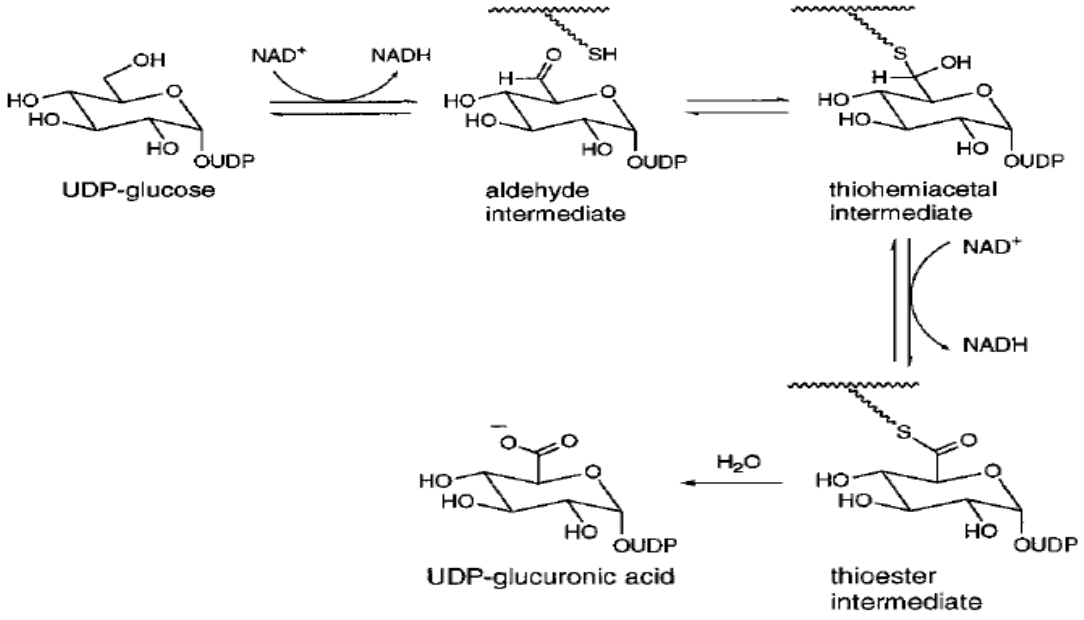

Figure 5. Proposed mechanism of the reaction catalyzed by UDP-glucose dehydrogenease. Wavy lines indicate amino acid residues within the active site of the enzyme. [138]

1.3.8 Sugar metabolism and capsular polysaccharide production

RegM, a homologue of the staphylococcal catabolite control protein CcpA, which is involved in the regulation of sugar metabolism pathway, has been shown to affect transcription of the *cps* locus, which suggests that a carbon source might also influence capsular expression [132].

Two proteins that are involved in sugar metabolism and whose genes are located outside the capsule operon have been shown to affect CPS production. Pgm is the phosphoglucomutase that catalyses the conversion of glucose-6-phosphate to glucose-1-phosphate [139], and GalU is a glucose-1-phosphate uridylyltransferase that catalyses the formation of uridine diphosphate-glucose (UDP-Glc) from glucose-1-phosphate [116]. Mutants of *S. pneumoniae* in which either the *galU* or *pgm* gene

was disrupted produced almost no CPS and exhibited growth defects [140, 116]. Additionally, pneumococcal strains in which the *pgm* gene had defined point mutations that significantly reduced but did not eliminate enzymatic activity still produced reduced amounts of CPS, even though the mutants no longer exhibited growth defects [141]. Both Pgm and GalU are required for the synthesis of UDP-Glc, which is a precursor for the biosynthesis of all 91 pneumococcal CPS types, as well as other cellular structures, such as teichoic acid. Thus, limiting the supply of this precursor would be expected to impact heavily upon CPS production in the pneumococcus. Therefore, indirect modulation of CPS production by controlling the availability of precursors or co-factors could be one of the regulatory mechanisms that are used by *S. pneumoniae.*

1.3.9 Capsule genes may also be important for bacterial metabolism

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 [135, 142, 143]. Also there is evidence that in *C. albicans* glycosyltransferases (enzymes involved in polysaccharide biosynthesis) can have multiple cellular functions [144].

In frame *ugd* deletion mutants of *S. pneumoniae* D39 (*csp2K* mutants) were generated by Xayarath et al. [112]. In contrast to the large, glossy colonies of the encapsulated D39 parent, all of the *cps2K* mutants exhibited a small, rough colony morphology. Microscopic observation revealed fewer bacteria per chain for the mutants. Thus, an overall lower number of bacteria were present in each colony, suggestive of a possible growth defect. Further no surface localized capsule was detectable by electron microscopy. In the *cps2K* mutants the levels of both sugars (hexose and methylpentose) were approximately 5% of the parental levels [112].

Lack of the terminal GlcUA in *cps2K* deletion mutants of the side chain alters the ability to transfer the type 2 capsule to the cell wall. This lack of cell wall polymer in the *cps2K* mutants could reflect a requirement for recognition of GlcUA by one or more enzymes in the capsule pathway, an alteration in the secondary structure of the polymer such that it no longer serves as a substrate for one or more enzymes, or an insufficient level of polymer substrate for transfer. The *cps2K* mutants were unchanged with regard to Cps2D production and tyrosine phosphorylation, and thus

this system was not responsible for the observed reduction in capsule levels or the failure to transfer polymer to the cell wall. The absolute requirement for GlcUA may therefore lie with the enzyme(s), which have not yet been defined, necessary for transfer of the polymer from undecaprenyl-phosphate (Und-P) to the cell wall [112].

In *S. pneumoniae* strain D39 knockout of the entire capsule operon induced a prolonged lag phase in Todd Hewitt broth with yeast extract (THY medium) and normal growth could be restored by backtransformation of the capsule genes [145]. During the lag phase, the capsule gene mutant showed a slight temporary increase of the optical density (OD) before entering the phase of exponential growth. This suggests that cells started to grow initially, but were then confronted with limiting conditions. These may have been compensated for by some adaption of the bacterial cell, e.g. activation of an alternative metabolic pathway. The growth deficiency in the capsule operon mutant could be overcome by fetal bovin serum (FBS) supplementation. This indicates that FBS contains a rapid inducer for an alternative pathway and/or offers a carbon source or glucogenic amino acid that can be metabolized immediately by the cell without activation of an appropriate metabolic pathway [145]. Therefore capsule gene products play not only a role for the biosynthesis of the capsular polysaccharide, but also contribute to metabolic pathway(s) required for growth.

Recently, it was shown that serotypes differ in the length of their lag phase during growth in vitro [102]. Also a long lag phase was preferentially seen in serotypes with high invasive potential, whereas serotypes associated more with colonization tended to have shorter lag phase. The prolonged lag phase can be compensated for with serum supplementation [102]. We hypothesize that expression of some capsule genes diverts sugars used for capsule biosynthesis from the central metabolism, but some capsule gene products may also contribute enzymes with multiple functions to the central metabolism. This interplay between capsule gene products and the central metablism is likely to influence bacterial characteristics, such as growth.

2. Aim of this thesis

In this thesis two main areas were of interest:

2.1 Heteroresistance to penicillin in *Streptococcus pneumoniae*

Heteroresistance is an established, but poorly characterized phenomenon of clinical importance. To our knowledge, heterogeneous resistance to penicillin in *S. pneumoniae* has not been studied so far. Heteroresistance is of clinical importance because it may be missed during routine antibiotic resistance testing and may lead to therapeutic failure in pneumococcal infection. Also, heteroresistance may be a possible pathway employed by bacteria to evade antibiotic selection pressure and to evolve to higher resistance. The knowledge gained from this study will add to the understanding of pneumococcal antibiotic resistance and the phenomenon of heteroresistance.

Hypotheses: Heteroresistance to penicillin in *S. pneumoniae* does exist and:

a) can convert into a homogeneous (homotypic) resistance phenotype
b) depends on a combination of mutated *pbp1a, 2b*, and *2x* genes
c) mediates cross-heteroresistance to other cell wall active antibiotics
d) is not due to mutator phenotype

Aim: We will test whether:

a) heteroresistance to penicillin does exist in *S. pneumoniae*
b) conversion to homotypic resistance occurs in resistant colonies selected from population profile analysis of a strain with heteroresistance to penicillin.
c) heteroresistance depends on a combination of mutated *pbp1a, 2b*, and *2x* genes
d) heteroresistance requires overexpression of mutated *pbp1a, 2b*, and *2x* genes
e) heteroresistance occurs in the penicillin susceptible laboratory strain R6 upon transformation with the *pbp1a, 2b*, and *2x* genes from a heteroresistant strain.
f) Population analysis profile of heteroresistant strains reveal heteroresistance also to cephalosporine and vancomycin.
g) the spontaneous mutation rate of two heteroresistant strains shows a mutator phenotype.

Strategy: Clinical isolates, which showed an inner zone of hemolysis during testing MIC by Etest method will be analyzed for the presence of heteroresistance:

a) Population analysis profile (PAP) will be performed. Colonies growing at antibiotic concentrations above the MIC of the strain, as determined by Etest will be collected. Colonies will be analyzed for their MIC, PAP and the stability of the MIC upon repeated subcultures in antibiotic free medium.
b) *pbp*'s of strains with heteroresistance will be sequenced and PBP protein profile will be analyzed.
c) The laboratory strain R6 will be transformed with the *pbp1a, 2b*, and *2x* genes, individually and combined by second- and third-step transformation. Transformed colonies will be analyzed for heteroresistance by PAP.
d) PAP will be performed exposing the strains to cephalosporin (ceftriaxon) and vancomycin.
e) The mutation rate to rifampicin resistance will be measured.

2.2 Roles of *Streptococcus pneumoniae* polysaccharide capsule genes in growth

The polysaccharide capsule is the major virulence factor of *S. pneumoniae*. The capsule mediates its effect by protecting the bacteria from phagocytosis.

2.2.1 Influence of capsule gene *ugd* on growth and sugar metabolism in D39 serotype 2

Preliminary data showed that deletion of part or the entire capsule operon in the laboratory strain D39 (serotype) induces a prolonged lag phase in bacterial growth [145]. A likely explanation for the delayed growth of capsule gene mutants is that capsule gene products play not only a role for the biosynthesis of the capsular polysaccharide, but also contribute to metabolic pathways required for growth. Upon deletion of capsule genes the bacterial cell must activate an alternative metabolic pathway to be able to grow, which results in a growth delay.

Hypothesis: Capsule gene products of *S. pneumoniae* also influence bacterial growth, because capsule gene products have multiple functions in the bacterial metabolism besides their role in polysaccharide biosynthesis. Candidate capsule genes are the UDP-glucose-dehydrogenase gene (*ugd*), which catalyzes the

synthesis of glucuronic acid, and the *rml* genes (*rmlA, rmlB, rmlC, rmlD*), which catalyze the synthesis of rhamnose.

Aim: We will test:

a) whether knocking out the *ugd*, *rmlA, rmlB, rmlC,* or *rmlD* genes in the *S. pneumoniae* laboratory strain D39 (serotype 2) delays growth.
b) whether delayed growth is compensated by restoring the respective gene function.
c) whether delayed growth is due to the activation of an alternative metabolic pathway.
d) whether strains with delayed growth show impaired cell wall integrity.

Strategy: Experiments will be done with the *S. pneumoniae* laboratory strain D39 (serotype 2):

a) construct single gene knockout mutants for *ugd, rmlA, rmlB, rmlC,* and *rmlD*. Gene deletions must be in frame in order to allow for the expression of the downstream genes. Alternatively, we may try to block the respective gene function by siRNA [146].
b) substitute the deleted gene in the single gene mutants and in mutants lacking the whole capsule operon, by inserting the functional gene outside the capsule operon, for example into the *lacE* locus.
c) perform growth curves by offering the cells different carbon sources and by culturing cells in the supernatant of a midlog culture of mutants with delayed growth in order to test for the presence of an exoenzyme released through the activation of an alternative pathway.
d) perform protein analysis of the cell membrane/wall on mutants with altered growth.
e) test the cell wall integrity by measuring spontaneous and antibiotic induced lysis.

2.2.2 Does capsule switch transform colonization characteristics?

Pneumococcal serotypes differ in their in vitro growth properties. Typical invader serotypes have significantly longer lag phases than typical colonizer serotypes. The prolonged lag phase can be compensated by serum supplementation [102]. The

expression of some capsule genes may divert sugars used for capsule biosynthesis from the central metabolism but some capsule gene products may also contribute enzymes with multiple functions to the central metabolism. This interplay between capsule gene products and the central metabolism is likely to influence bacterial characteristics, such as growth.

Hypothesis: The ability of *S. pneumoniae* to colonize the nasopharynx is influenced by the interplay between capsule gene products and the central metabolism:

a) the expression of the capsule gene cluster of serotypes with a high colonization prevalence (colonizer) allows for growth in the nasopharynx.
b) the expression of the capsule gene cluster of serotypes with low colonization prevalence, but relative high frequency of invasive disease (invaders) limits growth in the nasopharynx.

Aim: We will test whether capsule switch between colonizer and invader *S. pneumoniae* serotypes transforms characteristics from an invader phenotype into a colonizer phenotype and vice versa in terms of in vitro growth, in vitro adherence and in vivo colonization in an animal model.

Strategy: Four invader or colonizing serotypes, clinical isolates of *S. pneumoniae* will be chosen from our strain collection (two isolates for each group).

a) isogenic capsule exchange mutants between invader and colonizer serotypes will be constructed.
b) capsule exchange mutants of strain D39 will be constructed for each of the four serotypes.
c) capsule exchange mutants will be tested for changes of growth characteristics, in vitro adherence to respiratory epithelial cells and colonization capacity in an animal model of nasopharyngeal colonization.

3. Heteroresistance to Penicillin in *Streptococcus pneumoniae*

3.1. Publication

Brigitte Morand and Kathrin Mühlemann. Heteroresistance to Penicillin in *Streptococcus pneumoniae.* Proc Natl Acad Sci USA 2007 Aug 28:104(35): 14098-14103. Epub 2007 Aug 17.

Heteroresistance to penicillin in *Streptococcus pneumoniae*

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Heteroresistance to β-lactam antibiotics has been mainly described for staphylococci, for which it complicates diagnostic procedures and therapeutic success. This study investigated whether heteroresistance to penicillin exists in *Streptococcus pneumoniae*. Population analysis profile (PAP) showed the presence of subpopulations with higher penicillin resistance in four of nine clinical pneumococcal strains obtained from a local surveillance program (representing the multiresistant clones ST179, ST276, and ST344) and in seven of 16 reference strains (representing the international clones Spain^{23F}-1, Spain^{9V}-3, Spain^{14}-5, Hungary^{19A}-6, South Africa^{19A}-13, Taiwan^{23F}-15, and Finland^{6B}-12). Heteroresistant strains had penicillin minimal inhibitory concentrations (MICs) (for the majority of cells) in the intermediate- to high-level range (0.19–2.0 μg/ml). PAP curves suggested the presence of subpopulations also for the highly penicillin-resistant strains Taiwan^{19F}-14, Poland^{23F}-16, CSR^{19A}-11, and CSR^{14}-10. PAP of bacterial subpopulations with higher penicillin resistance showed a shift toward higher penicillin-resistance levels, which reverted upon multiple passages on antibiotic-free media. Convergence to a homotypic resistance phenotype did not occur. Comparison of two strains of clone ST179 showed a correlation between the heteroresistant phenotype and a higher-penicillin MIC and a greater number of altered penicillin-binding proteins (PBP1a, -2b, and -2x), respectively. Therefore, heteroresistance to penicillin occurs in international multiresistant clones of *S. pneumoniae*. Pneumococci may use heteroresistance to penicillin as a tool during their evolution to high penicillin resistance, because it gives the bacteria an opportunity to explore growth in the presence of antibiotics before acquisition of resistance genes.

Penicillin resistance has emerged in *Streptococcus pneumoniae* within a few decades after the introduction of penicillin and has spread successfully worldwide. A relatively small number of resistant clones have been mainly responsible for the current international resistance epidemiology (1, 2). Determinants of pneumococcal penicillin resistance are mutations in high-molecular-weight class A and B penicillin-binding proteins (PBP), which probably accumulate in commensal streptococci and are then transformed into pneumococci (3, 4). There is increasing evidence that auxiliary genes are needed for the expression of pneumococcal penicillin resistance, such as the *murMN* genes and the ciaH/R two-component system (5–8). For genetic and statistical reasons, it is unlikely that the different resistance components are acquired during a single transformation or mutation event. However, it is unknown whether there is a defined chronological order or whether single components can provide a selection advantage to the bacterial cell on the way to resistance.

Heteroresistance may play a role in this evolutionary process. The term has not yet been clearly defined, but it is usually understood as the presence of one or several bacterial subpopulations at a frequency of 10^{-7} to 10^{-3}, which can grow at higher antibiotic concentrations than predicted by the minimal inhibitory concentration (MIC) for the majority of cells. Most studies have concentrated on heteroresistance to methicillin and vancomycin in staphylococci (9–15). There are some reports of other pathogens, such as heteroresistance to rifampicin in mycobacteria (16), to vancomycin in enterococci (17), to colistin in *Acinetobacter* spp (18), and to fluconazole in *Cryptococcus neoformans* (19).

Heteroresistance creates clinical and diagnostic problems, but it is also intriguing from an evolutionary standpoint. Heteroresistance may give the microorganism the opportunity to explore growth at higher antibiotic concentrations without paying the fitness costs that may be associated with the acquisition of resistance genes, such as altered PBP genes (20). Heteroresistance may therefore serve as a tool used by bacteria during evolution to resistance.

In this study, we searched for heteroresistance to penicillin in *S. pneumoniae*. We were motivated by a phenomenon observed during resistance testing of clinical pneumococcal isolates collected within a nationwide surveillance program (21). Upon determination of the penicillin MIC by the Etest method (AB Biodisk, Solna, Sweden), some strains exhibited an inner zone of hemolysis but no visible bacterial growth. We hypothesized that this inner hemolysis zone may indicate the growth of a subpopulation of bacteria with higher resistance levels.

Results

Antibiotic Susceptibility Testing. Penicillin MICs determined by Etest of the study strains are shown in Table 1 (22, 23). Interpretation of penicillin Etests for strains 106.44, 110.58, 208.39, and 304.80 was ambiguous because of an inner zone of hemolysis without visible bacterial growth of appreciable diameter (>5 mm) (Fig. 1*A*). For example, for strains 208.39 and 304.80, interpretation of the Etest based on the bacterial lawn yielded an MIC of 0.008 μg/ml, whereas the MIC was 0.75 μg/ml based on the width of the hemolysis zone. For strains 110.58 and 106.44, MIC values based on the bacterial lawn were 0.012 and 0.5 μg/ml, respectively. However, the hemolysis zone indicated a MIC of 0.19 and 1.0 μg/ml, respectively. MIC values obtained from macrobroth dilution were even higher than the MIC read from the hemolysis zone of the Etest; it was 1.29 μg/ml for strain 208.39, 1.44 μg/ml for strain 304.80, 0.75 μg/ml for strain 110.58, and 1.0 μg/ml for strain 106.44. These observations suggested the presence of subpopulation(s) with higher resistance level(s) in strains 106.44, 110.58, 208.39, and 304.80. Subcultures taken from the hemolysis zone and inoculated on Columbia sheep blood agar (CSBA) plates yielded bacterial growth in all four strains. MIC values obtained from these subcultures were comparable to the values for the majority of the population (0.75 μg/ml for strain 208.39, 0.75 μg/ml for strain

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Abbreviations: MIC, minimal inhibitory concentration; MLST, multilocus sequence typing; PAP, population analysis profile; PBP, penicillin-binding protein; PFGE, pulsed-field gel electrophoresis; CSBA, Columbia sheep blood agar.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EF989125–EF989160).

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Table 1. Strains of *S. pneumoniae* used in this study

Strain	Serotype	MLST	MIC penicillin,* μg/ml	Reference or source
Swiss clinical strains				
111.46	19F	177	0.064	This study
207.41	19F	179	0.064	20
202.47	19F	177	0.094	20
202.58	1	305	0.064	This study
208.39	19F	276	0.75	This study
304.80	19F	179	0.75	20
106.44	nt	344	1.0	20, 21
110.58	nt	344	0.19	21
306.75	nt	1560	0.064	This study
Reference strains for international clones				
SP264	23F	81	2	Spain^{23F}-1 (ATCC 700669)
GM17	6B	90	2	Spain^{6B}-2 (ATCC 700670)
TL7/1993	9V	156	2	Spain^{9V}-3 (ATCC 700671
HUN663	19A	268	2	Hungary^{19A}-6 (ATCC 700673)
17619	19A	75	0.5	South Africa^{19A}-7 (ATCC 700674)
50803	6B	185	0.5	South Africa^{6B}-8 (ATCC 700675)
PN93/872/B	14	9	0.03	England^{14}-9 (ATCC 700676)
87—029055	14	20	8	CSR^{14}-10 (ATCC 700677)
91—006571	19A	175	4	CSR^{19A}-11 (ATCC 700678)
MS22	14	18	2	Spain^{14}-5 (ATCC 700902)
43362 FiB	6B	270	1	Finland^{6B}-12 (ATCC 700903)
51702	19A	41	2.0	South Africa^{19A}-13 (ATCC 700904)
TW31	19F	236	8.0	Taiwan^{19F}-14 (ATCC 700905)
TW17	23F	242	1–2	Taiwan^{23F}-15 (ATCC 700906)
Pol178	23F	173	8	Poland^{23F}-16 (ATCC BAA-343)
CS111	23F	37	0.12	Tennessee23F-4 (ATCC 51916)
Laboratory strains				
D39	2	128	0.006	22
R6	nt	128	0.026	22
QK25/010641	19F	nd	0.25	ATCC49619

*MIC determined by Etest.

304.80, 0.094 μg/ml for strain 110.58, and 1.0 μg/ml for strain 106.44). However, sampling of the hemolysis zone for technical reasons is not precise and is prone to give a mixture of subpopulations, including the subpopulation representative of the majority of cells. Therefore, population analysis profiles (PAPs) for penicillin resistance were obtained.

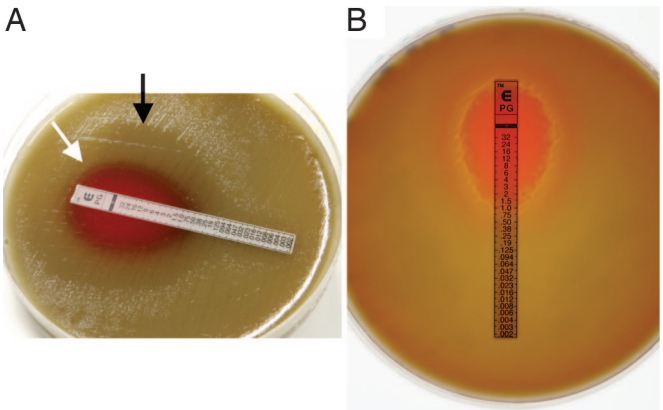

Fig. 1. Characteristics of heteroresistant *S. pneumoniae* strains in the penicillin Etest. (*A*) Typical example of the zone phenomenon observed in the penicillin Etest for some *S. pneumoniae* strains with heteroresistance to penicillin (see also Table 2). The black arrow indicates the end of the bacterial lawn, and the white arrow points to the end of the hemolysis zone. (*B*) Satellite colonies observed in the penicillin Etest for some *S. pneumoniae* strains with heteroresistance to penicillin.

PAP. PAP confirmed the presence of subpopulations with higher penicillin resistance levels for Swiss strains 106.44, 110.58, 208.39, and 304.80 (Table 2 and Fig. 2*A*). The character of the PAP curve for strains 208.39 and 304.80 suggested the presence of several subpopulations with different penicillin resistance levels at frequencies between 10^{-3} and 10^{-5}, in accordance with the class II heteroresistance pattern described before for methicillin-resistant staphylococci (11). The PAPs for strains 110.58 and 106.44 were closer to the picture of class III with one subpopulation at 10^{-4} to 10^{-5} or 10^{-6}, respectively. No heteroresistance to vancomycin could be observed for the Swiss strains selected for this analysis (data not shown).

Based on these results for the local Swiss strains, PAP was also performed for 16 reference strains representing international pneumococcal clones. Seven of these clones exhibited a PAP result consistent with heteroresistance to penicillin (Fig. 2 *B* and *C*). These included strains Spain^{23F}-1, Spain^{9V}-3, Spain^{14}-5, Hungary^{19A}-6, South Africa^{19A}-13, Taiwan^{23F}-15, and Finland^{6B}-12. The PAP curves were compatible with class II heteroresistance. During Etest for penicillin, these seven strains did not exhibit a large hemolysis zone as described for the heteroresistant Swiss strains. However, satellite colonies could be observed for strains Hungary^{19A}-6 and Taiwan^{23F}-15.

PAP curves also suggested the presence of subpopulations for the highly penicillin-resistant strains Taiwan^{19F}-14, Poland^{23F}-16, CSR^{19A}-11, and CSR^{14}-10 (Fig. 2 *B* and *C*), and all strains exhibited satellite colonies in Etests for penicillin (data not shown). However, the relative range of penicillin concentrations spanned by the plateau was relatively narrow (≤2-fold increase in penicillin concentration).

Table 2. Population analysis profile for penicillin resistance of *S. pneumoniae* strains

Strain	MLST type	MIC,† μg/ml	Frequency of cells with higher resistance‡	Highest penicillin concentration with bacterial growth,§ μg/ml
111.46	177	0.064	None	0.05
207.41	179	0.064	None	0.08
202.47	177	0.094	None	0.08
202.58	305	0.032	None	0.04
208.39¶	276	0.75	10^{-3} to 10^{-5}	0.75
HOM*1		1.0	10^{-3} to 10^{-6}	1.75
HOM*1p		1.0	10^{-3} to 10^{-5}	1.25
HOM*2		2.0	10^{-3} to 10^{-5}	1.50
HOM*3		3.0	10^{-3} to 10^{-5}	2.4
304.80¶	179	0.75	10^{-3} to 10^{-5}	1.0
HOM*1		1.0	10^{-2} to 10^{-4}	1.5
HOM*1p		0.75	10^{-3} to 10^{-6}	1.25
HOM*2		4.0	10^{-3} to 10^{-5}	2.1
HOM*3		2.0	10^{-3} to 10^{-5}	2.1
106.44¶	344	1.0	10^{-4} to 10^{-5}	1.0
HOM*1		1.0	10^{-4} to 10^{-5}	1.5
HOM*1p		1.0	10^{-2} to 10^{-5}	0.9
HOM*2		3.0	10^{-2} to 10^{-3}	1.5
HOM*3		3.0	10^{-2} to 10^{-4}	1.5
110.58	344	0.19	10^{-6}	0.6
306.75	1560	0.064	None	0.05
Spain^{23F}-1	81	2.0	10^{-2} to 10^{-3}	2.0
Spain^{6B}-2	90	1.5	None	1.25
Spain^{9V}-3	156	2.0	10^{-3} to 10^{-6}	2.1
Hungary^{19A}-6	268	1.0	10^{-3} to 10^{-5}	2.5
South Africa^{19A}-7	75	0.38	None	0.325
South Africa^{6B}-8	185	0.25	None	0.325
England^{14}-9	9	0.032	None	0.03
CSR^{14}-10	20	12	None	14.0
CSR^{19A}-11	175	12	None	8.0
Spain^{14}-5	18	2	10^{-5}	3.5
Finland^{6B}-12	270	0.75	10^{-3}	1.0
South Africa^{19A}-13	41	3.0	10^{-3}	3.0
Taiwan^{19F}-14	236	8.0	None	6.0
Taiwan^{23F}-15	242	1.5	10^{-3} to 10^{-6}	2.0
Poland^{23F}-16	173	16	None	8.0
Tennessee^{23F}-4	37	0.125	None	0.1
D39	128	0.023	None	0.02
R6	128	0.026	None	0.02
QK25/010641	Not determined	0.38	None	0.32

†MIC determined by Etest.
‡Frequency of subpopulations with higher penicillin-resistance levels as determined by population profile analysis (see also Figs. 2 and 3).
§The highest penicillin concentration at which bacterial growth could be observed during population profile analysis.
¶HOM* strains were generated by propagation of cells from subpopulations with higher penicillin-resistance level as explained in *Materials and Methods*.

Stability of Heteroresistance to Penicillin. At least three repetitions of PAP for each strain starting from the frozen bacterial stock documented a remarkable stability of the heteroresistant phenotype [for details, see supporting information (SI)]. The resistance behavior of subpopulations with higher resistance levels was analyzed for strains 208.39, 304.80, and 106.44. Single colonies were picked from PAP plates with the highest or second-highest penicillin concentrations showing bacterial growth and subjected to PAP immediately or after 10 passages on antibiotic-free agar plates. The resulting progeny strains were called HOM*1 and HOM*1p, in reminiscence of the work done in staphylococci (11). HOM*1 generations showed a shift of the PAP curve to slightly higher penicillin concentrations, but the shape or class of the PAP curve was essentially preserved (Fig. 3). Passage of HOM*1 strains 10 times without antibiotics shifted the PAP curve back to the range of penicillin concentrations of the parental strain for 304.80 and 106.44 but not for strain 208.39-HOM*1 (Fig. 3).

PAP of HOM*2 and HOM*3 strains (generated from HOM*1 and HOM*2 strains, respectively) showed the same trend as observed for HOM*1 strains, with a gradual shift of the curves toward higher penicillin concentrations. Again, the overall shape or class of the curve was maintained, i.e., HOM* strains did not convert to a homogeneous resistance profile (Fig. 3). Interestingly, for HOM*2 and HOM*3 strains, satellite colonies appeared during Etest for penicillin (Fig. 1*B*).

PBP Profiles in HOM* Strains. To explore whether HOM* derivatives expressed the same PBP genes as their parent strains, the transpeptidase region of *pbp1a*, *-2b*, and *-2x* was sequenced for the strains 208.39, 304.80, 106.44, and 110.58 (GenBank accession nos. EF989125–EF989160). Numerous attempts to obtain sequences for the *pbp1a* fragment downstream of the conserved motif II for strain 110.58 and its HOM* derivatives were unsuccessful. Analysis of all other sequences showed that HOM*

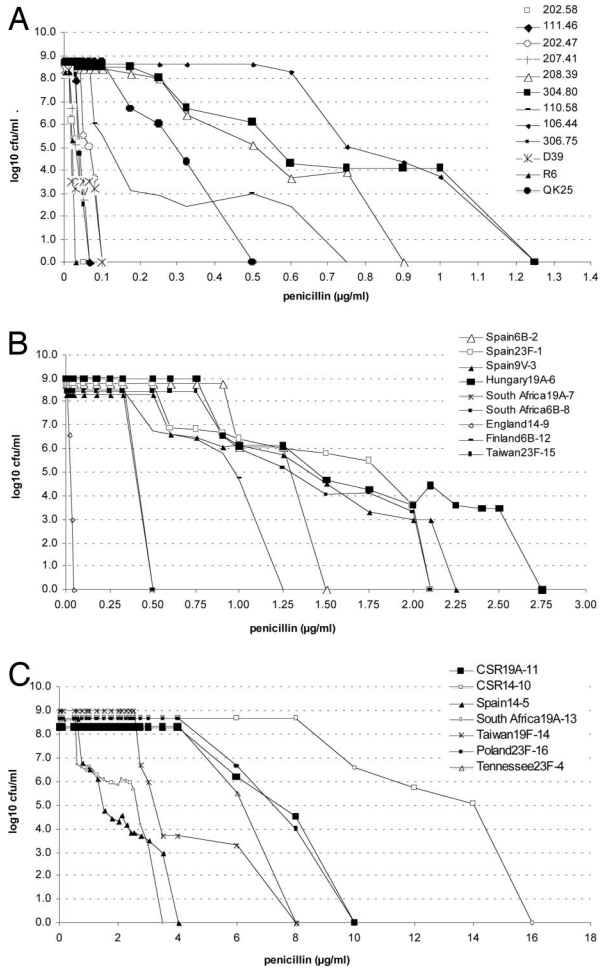

Fig. 2. PAP for *S. pneumoniae* strains (Table 1). The *x* axis indicates the penicillin concentration in micrograms per milliliter used to select supopulations with higher penicillin-resistance levels, and on the *y* axis, the frequency of bacterial cells is given as the logarithm to the base 10 of cfu per milliliter. (*A*) PAP for *S. pneumoniae* strains collected in Switzerland and laboratory strains (see also Table 1). (*B*) PAP for *S. pneumoniae* strains representative of international clones (see also Table 1). (*C*) PAP for *S. pneumoniae* strains representative of international clones (see also Table 1).

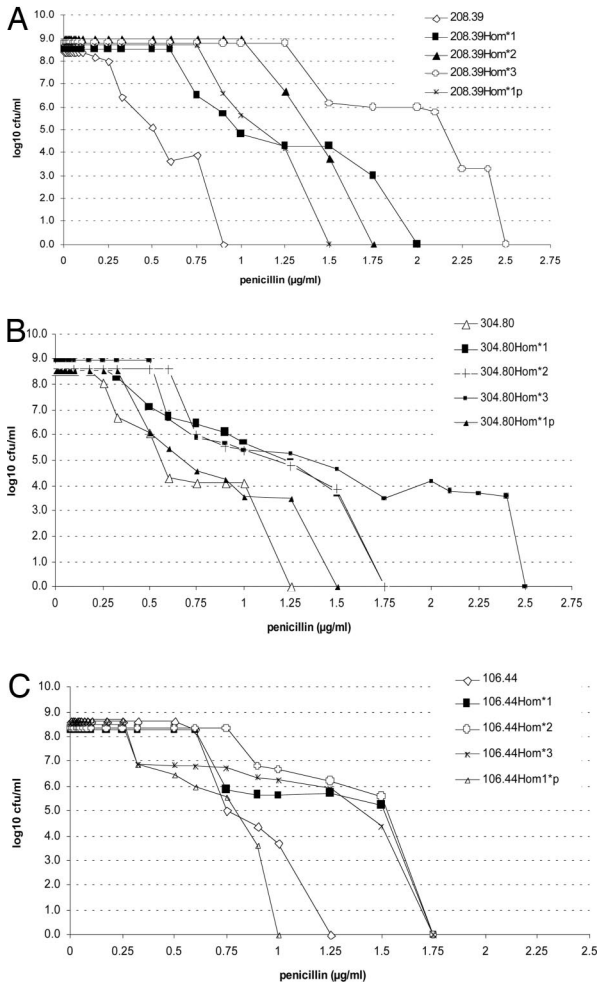

Fig. 3. PAP for the HOM* strains of three Swiss strains 208.39, 304.80, and 106.44 with heteroresistance to penicillin. HOM*1, HOM*2, and HOM*3 stands for derivatives of the respective strains obtained by selection of single colonies during successive PAP experiments. Colonies were selected from the plate with the highest or second-highest penicillin concentration showing bacterial growth. HOM*1p stands for a HOM1* strain that has been passaged 10 times on CSBA plates without antibiotics before repeating PAP. The *x* axis indicates the respective penicillin concentration in micrograms/milliliters used to select resistant subpopulations; on the *y* axis, the frequency of bacterial cells is given as the logarithm to the base 10 of cfu per milliliter.

derivatives carried the same *pbp1a*, *-2b*, and *-2x* genes as their parent strains.

Patterns of radiolabeled PBPs also confirmed that the HOM* strains of the heteroresistant strains 208.39, 304.80, 106.44, and 110.58 expressed the same PBPs as their parent strains (for details, see SI).

Colony Size. Strains with heteroresistance to penicillin exhibited a picture of varying colony size when grown on CSBA plates with or without penicillin, as demonstrated for strain 304.80 in Fig. 4. The range of colony diameters was wider for strain 304.80 than strain D39 with or without the presence of penicillin in agar plates. However, penicillin MIC values and PAP did not differ between small and large colonies (for details, see SI).

Phylogenetic Relationships Among Swiss Strains. Molecular typing was performed for the Swiss pneumococcal strains (Table 3). Some of these data have been reported (22, 23).

The serotype 19F strains 111.46, 202.47, 207.41, and 304.80 were phylogenetically related, because they all belong to the pulsed-field gel electrophoresis (PFGE) clone H (22, 24), and they belong to multilocus sequence typing (MLST) 177 or 179, which differ for the gki allele only (type 40 instead of type 4). By PFGE, strains 207.41 and 304.80 differed by two bands (data not shown). Interestingly, of these four related strains, only strain 304.80 exhibited heteroresistance to penicillin, but it also showed the highest penicillin MIC and the greatest number of altered *pbp* genes (Table 3).

Serotype 19F strain 208.39 with heteroresistance to penicillin was a member of the international resistant clone ST276 (25).

Strains 110.58 and 106.44 belonged to the nonencapsulated clone ST344, but their PFGE patterns differed by two bands (data not shown). Also, they showed mutated *pbp1a*, *-2b*, and *-2x* genes and differed in their gene sequence between each other (Table 3). Both strains exhibited heteroresistance to penicillin, but strain 110.58 with lower penicillin MIC had only one subpopulation at low frequency (Fig. 2*A*).

All four Swiss strains with heteroresistance to penicillin had mutations in all three *pbp* genes analyzed. Intriguingly, three of the four strains (208.39, 304.80, and 106.44) showed a high sequence homology for the *pbp2x* gene, and strains 304.80 and 106.44 also shared the *pbp1a* gene sequence.

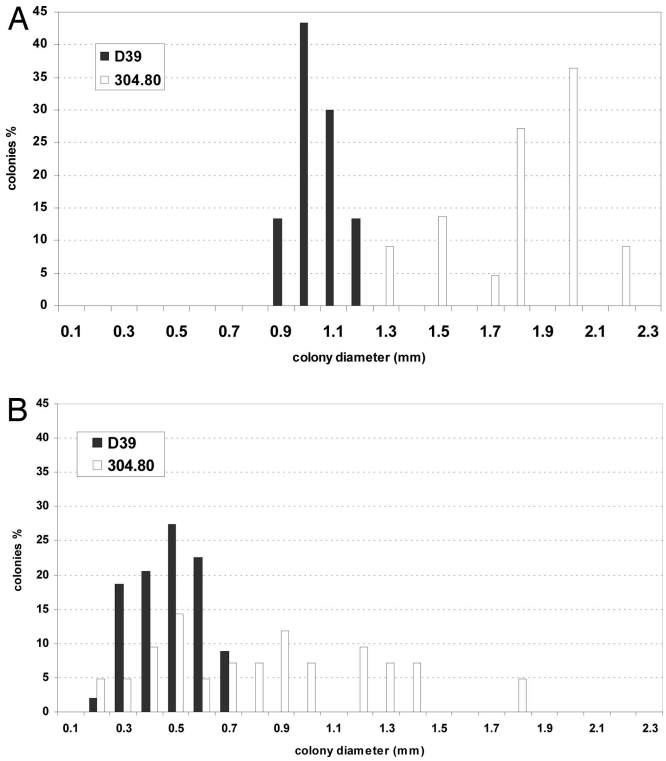

Fig. 4. *S. pneumoniae* strains with heteroresistance to penicillin exhibited visible heterogeneity of bacterial colony size when grown on CSBA plates with or without penicillin. This is demonstrated by comparing colony size diameters between the laboratory strain D39 and strain 304.80 grown on agar plates without antibiotics (*A*) and plates containing 0.02 μg/ml penicillin for strain D39 and 0.5 μg/ml penicillin for strain 304.80 (*B*). The *x* axis indicates the colony diameter in millimeters. The *y* axis gives the proportion of colonies with the respective colony diameter.

Discussion

Heteroresistance describes the existence of one or several subpopulations of bacterial cells with higher resistance levels than the majority of cells in a population profile analysis (26). Most observations on heteroresistance reported in the literature concern staphylococci resistant to methicillin, vancomycin, and/or inhibitors of teichoic acid synthesis (9–15). This study provides evidence for the existence of heteroresistance to penicillin in *S. pneumoniae*.

The results presented here for *S. pneumoniae* are well in line with reports on heteroresistance to methicillin in staphylococci. Pneumococcal subpopulations with higher resistance levels occurred at expected frequencies (10^{-3} to 10^{-6}). Other characteristics of heteroresistance, such as heterogeneity in colony size and growth of satellite colonies in the Etest, could also be observed. Similar to the findings for staphylococci, PAP results were remarkably stable for a given strain (11, 14, 27). *Staphylococcus aureus* with heteroresistance to methicillin have been categorized into four classes based on the shape of the PAP curves (11). The *S. pneumoniae* strains with heteroresistance to penicillin described here showed mainly a class II pattern, which stands for the existence of several subpopulations with different penicillin resistance levels. Only few strains fell into class III with one subpopulation only.

In methicillin-resistant *S. aureus*, propagation of highly resistant subpopulations in the presence of methicillin induced conversion from heterotypic to homotypic resistance (11, 14). Repeated propagation of HOM* strains for *S. pneumoniae* with heteroresistance to penicillin also showed a gradual increase in penicillin-resistance levels but only a trend of converting to a homotypic resistance pattern. Upon passage of HOM*1, strains in antibiotic-free strains tended to return to the penicillin-resistance phenotype of their parent strain.

All pneumococcal strains with heteroresistance to penicillin identified in this study belonged to well characterized international multiresistant clones (1, 25, 28). These strains had penicillin MIC levels for the majority of bacterial cells ranging between 0.19 and 2.0 μg/ml. That PAP of strains with very high penicillin MICs were only suggestive of heteroresistance to penicillin, although they showed satellite colonies in Etest, may be explained by a biological limit set to the pneumococcus for the highest-attainable MIC value.

The mechanisms involved in staphylococcal heteroresistance to methicillin are complex, and a considerable number of candidate genes have been implicated (9, 10, 12, 13, 15). This is likely also the case for heteroresistance to penicillin in *S. pneumoniae*. Three of the four Swiss strains with heteroresistance to penicillin shared the same *pbp2x* gene sequence but, in analogy to heteroresistance to methicillin in staphylococci, it seems unlikely that single PBP variants alone should be responsible for heteroresistance to penicillin. In preliminary experiments, we were unable to transfer heteroresistance to penicillin to other pneumococcal strains by transformation with the *pbp2x* gene of strain 304.80 (data not shown). Therefore, it seems more likely that known and/or unknown auxiliary resistance genes in concert with PBP variants are responsible for heteroresistance to penicillin in *S. pneumoniae*.

Table 3. Molecular typing of selected strains of *S. pneumoniae*

Strain	Serotype	MLST	Heteroresistance to penicillin	Penicillin MIC,* μg/ml	RFLP of PBP†		
					2x	2b	1a
111.46	19F	177	No	0.064	A	R6	R6
202.47	19F	177	No	0.094	Bs	R6 s	As
207.41	19F	179	No	0.064	A	R6	R6
304.80	19F	179	Yes	0.75	Cs	As	Bs
208.39	19F	276	Yes	0.75	Cs	R6s‡	Ds
110.58	Nt	344	Yes	0.19	Bs	Bs	Cs
106.44	Nt	344	Yes	1.0	Cs	Cs	Bs
D39	2	128	No	0.023	R6s	R6 s	R6s
R6	Nt	128	No	0.026	R6s	R6 s	R6s

*MIC determined by Etest.

†RFLP of PBP: RFLP profile analysis of PBP genes *1a*, *2b*, and *2x*; R6 means that the RFLP pattern for this strain was identical to, and A–D means the pattern showed one band or more difference from, the pattern observed for the laboratory strain R6; "s" indicates that the RFLP result was confirmed by DNA sequencing; sequences were called "identical" if nucleotide homology reached >96.5%.

‡The *pbp2b* DNA sequence in strain 208.39 revealed a fragment of 65-bp length immediately upstream of the conserved SXN motif II with 31% heterogeneity compared with the corresponding sequence in strain R6.

Heteroresistance to penicillin may be used by *S. pneumoniae* during evolution to higher-level penicillin resistance. This study included four phylogenetically related pneumococcal strains belonging to PFGE clone H or MLST ST177 and ST179, but only the strain with highest-penicillin MIC level and the greatest number of altered PBP genes was heteroresistant to penicillin. Earlier reports have described the plasticity of this clone in terms of penicillin-resistance levels and acquisition of *pbp* gene fragments (22, 24, 28). In addition, the study analyzed two nonencapsulated strains of MLST ST344. Both exhibited heteroresistance to penicillin. However, in the strain with lower-penicillin MIC (strain 110.58), subpopulations with higher resistance levels occurred at lower frequency (10^{-6}). It will be interesting to extend such analyses to other international pneumococcal clones.

In conclusion, this study provides evidence that heteroresistance to penicillin exists in *S. pneumoniae* and can be found in members of international multiresistant clones. We speculate that *S. pneumoniae* uses heteroresistance to penicillin during evolution to higher penicillin resistance. Heteroresistance may allow bacterial cells to explore growth at higher-penicillin concentrations without paying the fitness costs associated with the acquisition of new *pbp* gene fragments (20).

Materials and Methods

Bacterial Strains and Culture Conditions. Bacterial strains used for this study are presented in Table 1. The local Swiss strains of serotype 19F were part of a recent analysis of 108 clinical nasopharyngeal isolates of serotype 19F with an oxacillin disk diameter of <20 mm (1-μg disk) (22). Some of the nontypable isolates (106.44 and 110.58) have also been reported (22, 23). Reference strains for the international clones were kindly provided by Ralf Reinert (National Reference Center for Pneumococcus, Aachen, Germany). Control strains included the laboratory strain D39 (serotype 2), its spontaneous nonencapsulated derivative R6, and strain ATCC49619 (serotype 19F) (29).

The local Swiss pneumococcal strains were originally isolated from nasopharyngeal swabs from patients with acute respiratory tract infection (21, 28) and were stored after one *in vitro* passage at −80°C by using Protect bacterial preservers (Technical Service Consultants, Heywood, U.K.). For culture, bacteria were grown on CSBA plates at 37°C in a 5% CO_2-enriched atmosphere or in brain–heart infusion broth (Becton Dickinson, le Pont de Claix, France) containing 5% FCS (Biochrom, Berlin, Germany) at 37°C in ambient air.

Molecular Typing. MLST was done as described (30). The methods for PFGE, RFLP, and sequence typing of the genes encoding for PBP1a, -2b, and -2x have been reported (22). Membrane-enriched protein fractions were prepared, and radiolabeled PBPs were detected as described (31).

Antibiotic Susceptibility Testing. MIC were determined by the Etest method (AB Biodisk, Solna, Sweden) for penicillin and vancomycin. Macrobroth dilution was done for penicillin [Sigma–Aldrich (Buchs, Switzerland), P8721; 10 million units, Lot123K05211; Fluka, Buchs, Switzerland] in cation-adjusted Mueller–Hinton broth (Becton Dickinson), according to Clinical and Laboratory Standards Institute guidelines (32).

PAP. PAP was performed for resistance to penicillin and vancomycin according to the method described by Wootton *et al.* (26), with some adaptations. Briefly, bacteria were streaked out on CSBA plates and incubated for 24 h at 37°C in a 5% CO_2 atmosphere. An overnight culture was prepared by inoculating 3–10 colonies into 5 ml of brain heart infusion (BHI) containing 5% FCS in 15-ml tubes (Sarstedt, St. Gallen, Switzerland). The tubes were placed in a 37°C water bath for 9 h. One hundred microliters of the overnight culture was subcultured in 5 ml of BHI with 5% FCS and was grown to midlog phase (OD_{600} 0.7 encapsulated, OD_{600} 0.5–0.6 unencapsulated strains). Dilutions of this culture of 10^{-2} to 10^{-4} and 10^{-6} in PBS (pH 7.4) were prepared, and 100 μl was spiral-plated on Müller–Hinton broth (MHB) agar plates (BioMérieux, Geneva, Switzerland) with 5% sheep blood containing penicillin concentrations ranging from 0 to 18 μg/ml or vancomycin (vancomycin HCl, Sigma V2002, Lot 015K0825, Fluka) (concentrations 0.025, 0.125, 0.25, and 0.5 μg/ml). A dilution of the culture of 10^{-6} was spiral-plated onto a MHB plate with no antibiotic for determination of colony count. Colonies were counted by eye after 48 h of incubation at 37°C in 5% CO_2.

PAP for HOM*. Single colonies were selected from agar plates containing the highest or second-highest penicillin concentration at which bacterial growth was detectable. These colonies, called HOM*1 (11), were subcultured once (at a maximum, twice if necessary for sufficient growth) on CSBA plates without antibiotics and frozen at −80°C until PAP analysis. This procedure was repeated for HOM*1 strains yielding the HOM*2 generation and for HOM*2 strains generating HOM*3 strains. To test the stability of penicillin resistance, HOM*1 strains were subcultured for 8–10 passages on CSBA plates without antibiotics before PAP.

Determination of Colony Size. Colony size was determined during PAP experiments from CSBA plates after incubation for 24 h. The diameter of each colony was measured by using a stereomicroscope with an integrated 0.1-mm scale (Leica GZ4, Heerbrugg, Switzerland) at ×10 magnification.

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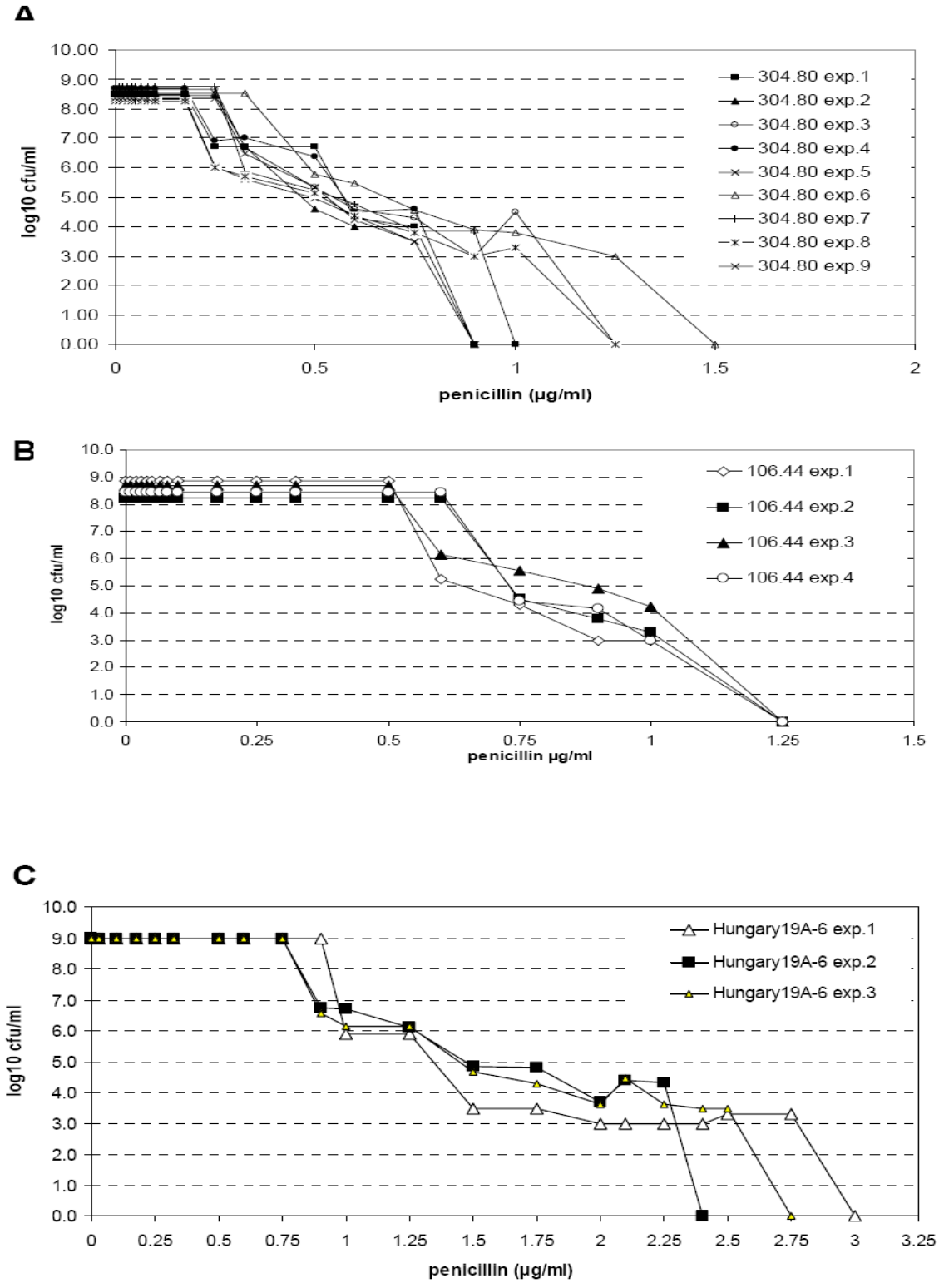
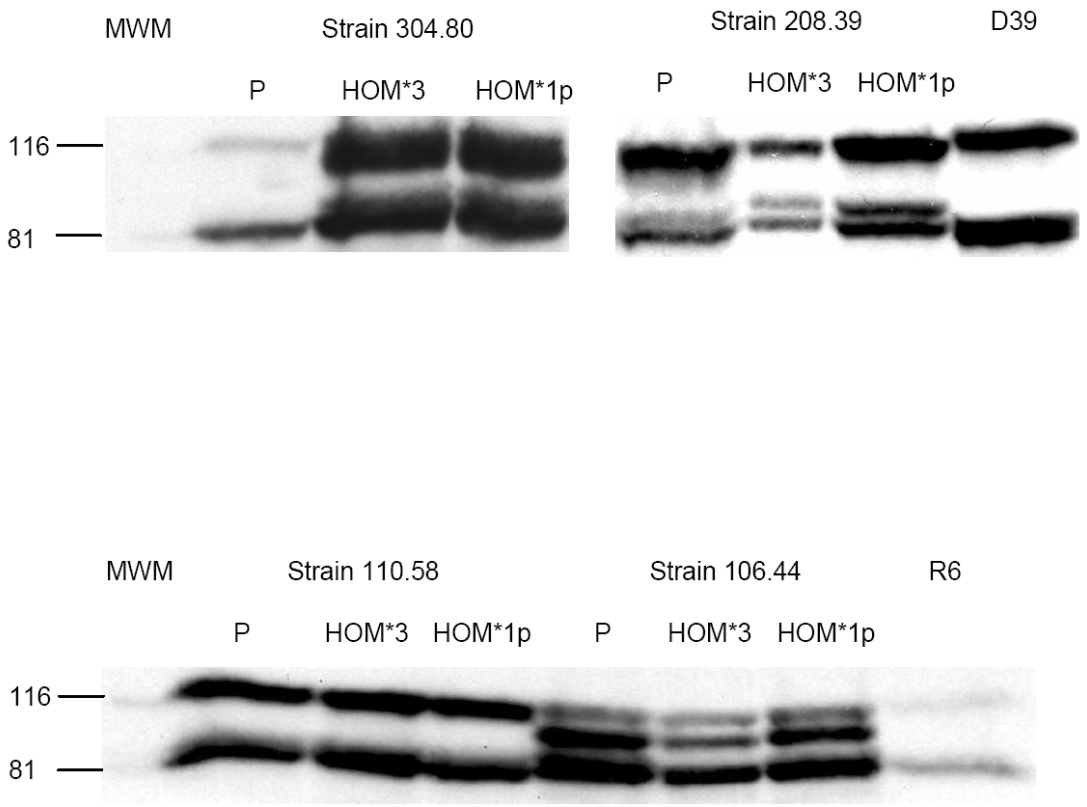
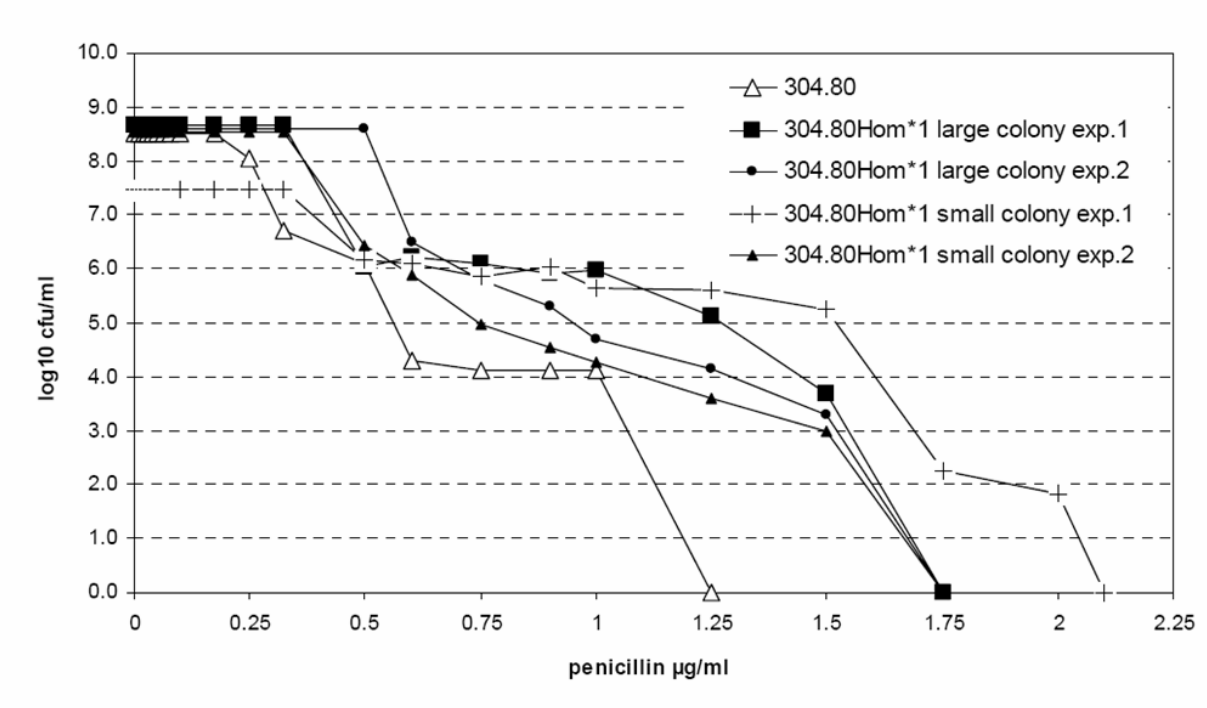
Supporting Information



SI Fig. 5. The stability of heteroresistance to penicillin was investigated by performing at least three separate population analysis profile (PAP) experiments for each strain. Here the results for three strains: A: 304.80, B: 106.44, and C: Hungary^{19A}-6 are shown



SI Fig. 6. Patterns of radiolabeled PBPs from four parent strains (208.39, 304.80, 106.44, and 110.58) with heteroresistance to penicillin and their HOM*1p and HOM*3 strains as well as the laboratory strains D39 and R6 are presented. The name of the strains are indicated at the tops of the lanes (P = parent strain). The molecular weight marker (MWM) indicates 116 and 81 kDa.



SI Fig. 7. Population analysis profiles of small and large colonies of *S. pneumoniae* strain 304.80 with heteroresistance to penicillin were compared.

3.2 Outlook

Our findings showed that heteroresistance to penicillin exists in *S. pneumoniae*. The underlying mechanism is however not known. Studies on heteroresistance in other bacterial species may help to raise hypotheses.

What can we learn from *Staphylococcus aureus?*

Most studies about heteroresistance have been performed in *Staphylococcus aureus* [73, 74, 77, 89, 147, 148]. Expression of resistance in *S. aureus* by altered PBP2' requires the efficient and correct synthesis of the peptidoglycan precursors. Genes involved in cell wall precursor formation and turnover, regulation, transport, and signal transduction may determine the level of resistance [76]. Screening for loss of methicillin resistance led to the identification of genes termed *fem* (factors essential for methicillin resistance) or *aux* (auxiliary) factors [149, 150]. Many of these *fem* or *aux* genes play a direct or indirect role in peptidoglycan biosynthesis and turnover, or have regulatory functions. However, none of these so called *fem* or *aux* factors has been shown to be the central mediator of heteroresistance in *S. aureus* [77].

Degrees of the heterogeneity and stability of the resistant clones of *Staphylococcus aureus* have been shown to be influenced by environmental factors [151]. A protective effect of NaCl on the susceptible subpopulation was observed when cells were exposed to nafcillin [152]. Heterogeneity was a thermosensitive property during growth of heterogeneous strains in the presence of methicillin. Cells were heterogeneous at 37°C and homogeneous, or close to homogeneous at 30°C [153]. Effect of low pH on methicillin resistance showed suppression, but not elimination of resistance. Slower growth was observed in acid medium, while methicillin resistance decreased to levels seen in sensitive strains [151].

The mechanism(s) of heteroresistance in *S. aureus* is unknown. However, indirect clues might be derived from experiments searching for the mechanism of methcillin or vancomycin resistance, since heteroresistance is often observed in such resistance phenotypes. The essential two-component regulatory system *yycF* (*vicR*) and *yycG* (*vicK)* was drastically up-regulated in a VISA strain as compared to a susceptible strain [87]. This up-regulation was due to an insertion element (IS256) in the predicted promoter region of *yycF* (*vicR*) and *yycG* (*vicK)* system, creating a potentially stronger hybrid promoter [87]. In another study a copy of IS256 inactivated

the *tcaA* gene, which seems to cause at least a part of the increase in vancomycin resistance [154]. *TcaR* has been postulated to be a member of transcriptional regulators and has recently been identified as a regulator of virulence in *S. aureus.*
Regulator gene function can be affected by mutation in different ways. Some mutations may cause complete inactivation of the regulator function or may partially inactivate it to various degrees depending on the position and nature of mutation (substitution to different amino acids) in the coding region. Microarray experiments revealed that a single mutation in response regulator *graR* alters the expression of more than 100 genes [86]. *GraR* belongs to a two component system *vraSR*, which is constitutively activated in VISA and heteroVISA but strongly repressed in vancomycin-susceptible *S. aureus*. vraSR turned out to be an upregulator of cell wall peptidoglycan synthesis. Its overexpression in VSSA strain increases MIC to the level, which are often found in hVISA (~2mg/l) but not to the level of VISA (defined as vancomycin MIC of ≥ 4mg/l) [155].
These findings suggest that specific regulatory systems control the occurrence of resistance, perhaps activated by triggers from external conditions.

It may be that heteroresistance in *S. pneumoniae* is due to a regulatory process, since propagation of HOM* strains did not show conversion to a homotypic resistance pattern, but a slight shift of MIC for the whole cell population was seen. Repeated passage of such HOM*1 strains without antibiotics led to a back-shift of the MIC to the range of the parental strain. This behaviour cannot be explained by stable mutations due to their frequency beyond the spontaneous mutations rate and its reversibility.

What can we learn from persisters?

Another phenomenon of bacteria for escaping antibiotic treatment has been observed decades ago and has been named persistence. Persisters are a small subpopulation of bacteria that survive lethal concentrations of antibiotic without detectable antibiotic resistance genes. This phenomenon was first reported for staphylococcal infections treated with penicillin [156] and has since been observed in many other bacterial species.
Despite being observed almost 60 years ago, the mechanism behind persistence remains a puzzle [157]. Currently, most studies on the mechanisms underlying

persistence have been performed with the *E. coli hipA*, mutant, which exhibits a higher frequency of persisters than wild-type strains [158 - 161].
It has been suggested that persistent bacteria are in a protected part of the cell cycle at the time of exposure to antibiotics or are able to adapt rapidly to the antibiotic stress [162]. It has also been proposed that those cells are in a dormant state or are unable to initiate programmed cell death [157, 163] or are aging bacteria [164].
Using microfluidic devices, persister bacteria were shown to either grow slowly or to not grow at all in the presence of antibiotics but to revert to normal growth on removal of antibiotics [165]. Furthermore, persister phenotype may be a pre-existing trait and not solely produced as a response to antibiotic treatment [166]. Balaban et al. could show that even before the antibiotic treatment persisters could be distinguished from the majority of cells by their reduced growth rate [167, 168]. Furthermore persister cells were able to spontaneously switch to fast growth and generate a population that is sensitive to the antibiotic. Different persister subpopulations may be distinguished, stationary phase induced persisters and spontaneous persisters [165].

Hetereoresistance and persister state show some similarities. The phenomenon affects subpopulations of bacteria and both seem to underlie phenotypic switch. A characteristic of persisters seems to be a slower growth rate. We could not find a difference between wild-type strains and HOM* strains for growth. However, growth of subpopulations on agar plates containing penicillin did require incubation for 48h which is longer than required by the majority of cells (24h). But, we cannot exclude that this was a direct effect of the antibiotic rather than an inherent characteristic of subpopulations. The technique of microfluidic device [167, 168] should deliver a useful technique for studying heteroresistant subpopulations.

What can we learn from other pathogens?

Heteroresistance in *Mycobacterium tuberculosis* seems to represents a one step event. In *Mycobacterium tuberculosis*, the majority of antibiotic resistance is caused by point mutations in a limited number of genes whose products either are the target of or activate the antituberculosis drug. These are typically one-step events immediately leading to a high level of resistance. Interestingly, *M. tuberculosis* susceptibility testing is unique in that a small number of resistant colonies is often seen and tolerated. For example, the widely accepted and WHO endorsed “1%

proportion method" allows for up to 1% resistant organisms in the primary isolate before the result of the susceptibility testing is declared to be resistant [169]. These findings suggest that mechanism of heteroresistance depends on the bacterial species and the specific antibiotic.

Phenotypic heterogeneity

Phenotypic heterogeneity is defined as the presence of individual cells in genetically homogeneous population that exhibit dissimilar phenotypes [170]. Heterogeneous response of bacterial populations to antibiotics and other stress factors has been linked more and more to their inherent nongenetic variability [171]. Heterogeneity provides a dynamic source of diversity, in addition to the diversity derived from genotype changes such as genome rearrangements and mutations. Microbial populations benefit by the creation of variant subpopulations that have the potential to be better equipped to persist during perturbation and to exploit new niches [172, 173]. Moreover, phenotypic heterogeneity does not invoke an irreversible commitment to the new state (that is, indefinite inheritability), so allowing rapid reversion to the original phenotype if appropriate. Therefore the benefits of heterogeneity to the fitness of the population can be readily envisaged. Although in vivo experiments to test the reality of such benefits are still lacking, modelling studies support the hypothesis, at least under certain conditions [174, 175].

Some mechanisms underlying heterogeneity are stochastic contributions to gene expression, progression through the cell cycle and biological rhythms, spontaneous epigenetic modifications, ageing linked or cell division, mitochondria activity and individual cell growth rates [173, 176]. These mechanisms are not necessarily independent of each other. Whether heteroresistant subpopulations will also form a chapter of phenotypic heterogeneity remains to be determined.

Clinical relevance of heterogeneity/heteroresistance

Heteroresistance in *S. aureus* leads to diagnostic problems. It has been shown, that heteroresistant MRSA are easily overlooked or misidentified [177]. Similarly vancomycin heteroresistant staphylococci are easily overlooked. The disk diffusion tests and several automated methods of susceptibility testing failed to detect glycopeptide-intermediate, possibly heteroresistant *S. aureus* [178]. Therefore,

development of appropriate methods to detect heteroresistant isolates would be necessary.

Future research in development of heteroresistance and heterogeneity

It would be particularly important to know whether heteroresistance influences the frequency of developing resistance under antibiotic therapy. Therefore, future research should define the growth conditions of pathogenic bacteria during infection which is needed to accurately predict the in vivo susceptibility to antibiotics and the potential for antibiotic resistance development. Antibiotic resistance genes can confer resistance when their expression is triggered by specific signals. These signals include the antibiotic itself [179], other types of antibiotics [180] and non-antibiotic compounds. Of particular concern is the induction of antibiotic gene expression by natural compounds that are present during infection. For instance, expression of *acrAB* [181] – a relevant multi-drug resistance pump of *Enterobacteriaceae* – is induced by bile salts that are present in the gastrointestinal tract [182].

Heterogeneity at the single–cell level is typically masked in conventional studies of microbial populations, which rely on data averaged across thousands or millions of cells in a sample. However, a recent surge of interest in the processes mediating cell-to-cell variability supported by methodological advances [183-186] may soon lead to new insight .

Future research on heteroresistance mechanism of *S. pneumoniae*

Heteroresistance is a natural phenomenon ensuring the adaptive strategy of bacteria to environmental changes. Further studies to elucidate the molecular mechanism of heteroresistance in *S. pneumoniae* should focused on the role of regulatory genes such as the two-component system CiaRH, which has been shown to play a role for pneumococcal penicillin resistance, or regulatory genes which are involved in cell wall biosynthesis. The regulation of genes that determine resistance levels may depend on control by global regulators whose fine-tuning may differ from strain to strain. Isolates from heteroresistant subpopulations should not be subcultured several times before testing, because of changing their gene expression pattern. Furthermore changes in gene expression levels by DNA microarrays should give an insight in different regulatory networks which are involved in development of antibiotic heteroresistance.

4. Roles of *Streptococcus pneumoniae* polysaccharide capsule genes in growth

4.1 Influence of capsule gene *ugd* on growth and sugar metabolism

4.1.1 Aim

We will test:

e) whether knocking out the *ugd* gene in the *S. pneumoniae* laboratory strain D39 (serotype 2) delays growth.
f) whether delayed growth is compensated by restoring the respective gene function.
g) whether delayed growth is due to the activation of an alternative metabolic pathway.
h) whether strains with delayed growth show impaired cell wall integrity.

4.1.2 Material and Methods

4.1.2.1 Principle of generating a single gene *ugd* knock out mutant and a *ugd* complemented strain

For the construction of single gene *ugd* knock out mutant the bicistronic cassette Janus was used [187, 188]. The Janus cassette was kindly provided by K. Trczinski, (Harvard School of Public Health, Boston, USA). Janus is a bicistronic cassette which permits selection both for its acquisition and its loss. Positive selection is based on kanamycin resistance (KanR). Negative selection is based on the resistance to streptomycin. Streptomycin resistance (SmR) is encoded by a spontaneous mutation in the gene *rpsL*, however, this mutation mediates a recessive resistance phenotype. The Janus cassette contains an intact *rpsL* and strains carrying the Janus cassette appear streptomycin susceptible (SmS), despite the presence of a resistance mutation in *rpsL* in the bacterial genome. Upon loss of Janus the strain becomes resistant to streptomycin and susceptible to kanamycin (KanS).

Construction of a single gene *ugd* knockout mutant: The capsule genes flanking *ugd* gene, *cpsJ* and *cpsP*, were amplified by PCR. By digestion with restriction enzymes and ligation with the ligase the PCR construct *cpsJ*-Janus-*cpsP* (Fig. 1A) was created. This was used to transformed into streptomycin resistant D39 (D39SmR)

(Fig. 1B) to create D39SmRΔ*ugd*::Janus (Fig. 1C). In a further step, the construct *cpsJ-cpsP* (Fig. 1D) was generated by fusion PCR to transform into D39SmRΔ*ugd*::Janus (Fig. 1E) to create an in frame single gene *ugd* knock out mutant, D39SmRΔ*ugd*::*cpsJ-cpsP* (Fig. 1E).

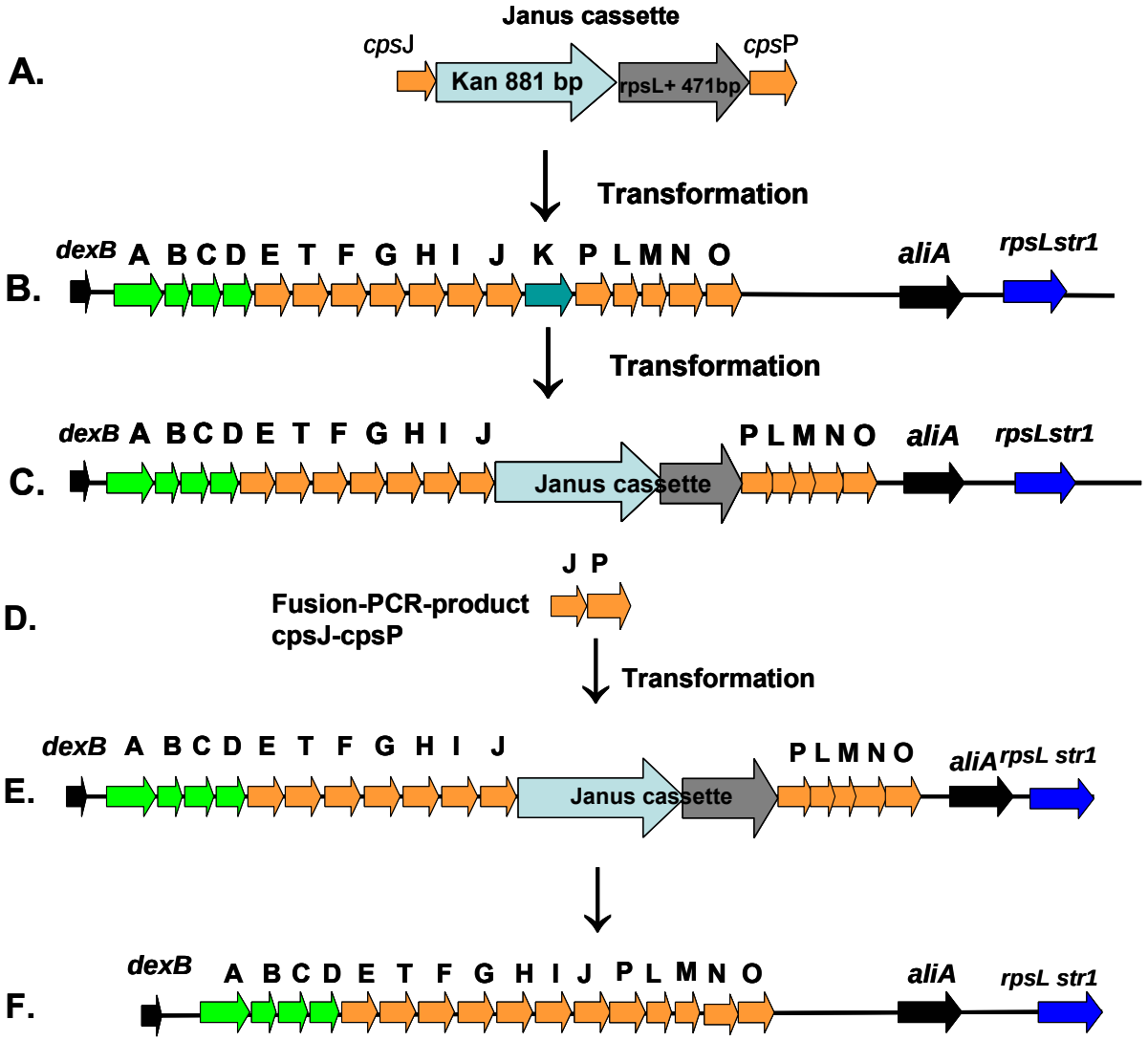


Fig. 1. Construction of a single gene *ugd* knockout mutant. A) *cpsJ*-Janus-*cpsP* PCR construct. B) D39SmR. C) D39SmRΔ*ugd*::Janus. D) *cpsJ-cpsP* fusion PCR product. E) D39SmRΔ*ugd*::Janus like C. F) D39SmR single gene *ugd* in frame knockout mutant, D39SmRΔ*ugd*::*cpsJ-cpsP.*

Construction of *ugd* complemented strain: The aim was to insert the functional *ugd* gene outside the capsule locus, into the *lacE* locus. By fusion PCR the *ugd* gene was ligated to the spectinomycin cassette (*aad9*). The first gene of the *lacE* operon upstream (*SPD0423*) and the last gene of the *lacE* operon downstream (*SPD0429*) were ligated by fusion PCR to the construct *aad9-ugd* (Fig. 2). It was planned to

transform this fusion PCR product into the in frame single gene *ugd* knockout mutant and into the mutant lacking the whole capsule operon to make D39Δ*ugd*::*cpsJ-cpsP*Δ*SPD0423-SPD0429*::*aad9-ugd* (Fig. 3B) and D39SmRΔ*cps*::*Janus*Δ*SPD0423-SPD0429*::*aad9-ugd* (Fig. 3C), respectively. By lack of the single gene *ugd* mutant, the fusion PCR product was transformed into *lacE* locus of D39 and into the mutant lacking the whole capsule operon.

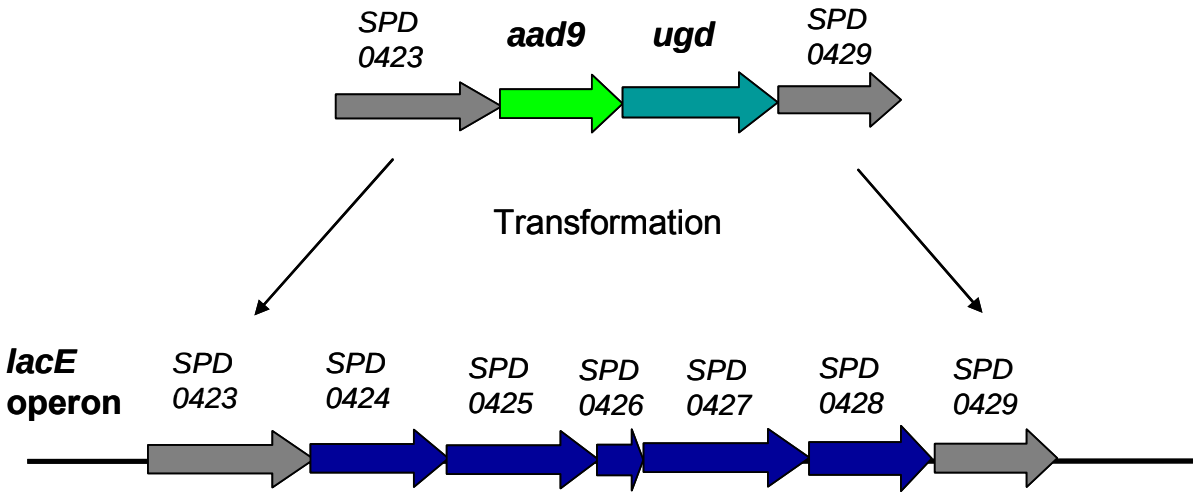


Fig. 2. Generation of the *SPD0423-aad9-ugd-SPD0429* PCR construct.

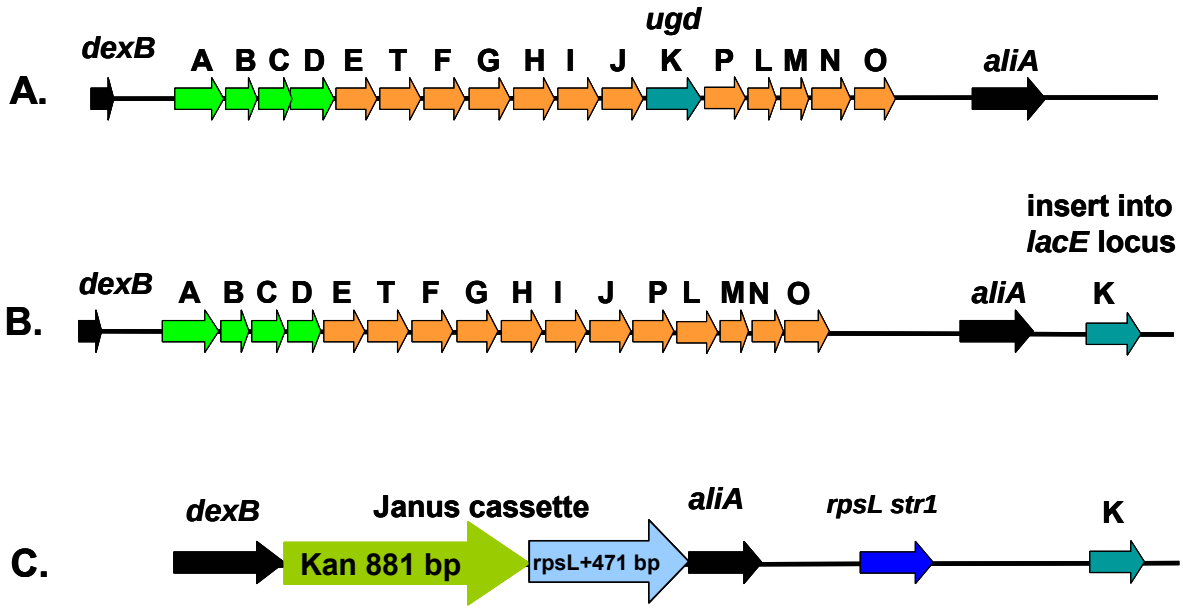


Fig. 3. Insertion of the *ugd* gene in the single gene *ugd* in frame knockout mutant outside the capsule operon (in *lacE* locus). A) D39. B) D39 *ugd* mutant with the *ugd* gene in *lacE* locus: D39Δ*ugd*::*cpsJ-cpsP*Δ*SPD0423-SPD0429*::*aad9-ugd* C) D39 without capsule with the *ugd* gene in *LacE* locus: D39SmRΔ*cps*::*Janus*Δ*SPD0423-SPD0429*::*aad9-ugd.*

4.1.2.2 Bacterial strains

Streptococcus pneumoniae strain D39 (serotype 2) [189] was kindly provided by Prof. Jeffrey Weiser (University of Pennsylvania, Philadelphia, PA). For growth curves in different media, additional strains were used, such as R6 (a spontaneous nonencapsulated derivative of D39) which was kindly provided by Prof. Philippe Moreillon (Centre Hospitalier Universitaire Vaudois, Lausanne). Three clinical isolates 204.76 (serotype 1), B112.30 (serotype 5) and 110.58 (nonencapsulated strain) were also used. Clinical isolates of *Streptococcus pneumoniae* were randomly selected from two nationwide surveillance programs collecting nasopharyngeal and invasive isolates [6, 16, 51].

4.1.2.3 Knocking out the *ugd* gene in strain D39

All primers were designed by using the Lasergene Software (DNASTAR Inc., Madison, Wis.). The genes *cpsJ* and *cpsP* were separately amplified with the forward primers cpsJ_BM47_F1, cpsP_C_F1_*Apa*I and with reverse primers cpsJ_B_B1_*Bam*HI, cpsP_BM54 (Annex, table A1) from the chromosomal DNA of D39 using Fast Taq DNA polymerase (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the manufacturer's instructions. Amplification was performed using the following cycling conditions: primary denaturation for 5 min at 94°C, followed by 30 cycles consisting of 94°C for 30 s, 50°C (annealing temperature) for 30 s and 72°C for 2 min (extension time) and then the last step for 10 min at 72°C. The forward primer for *cpsP* contained an *Apa*I terminus and the reverse primer of *cpsJ* a *Bam*HI terminus.

The Janus cassette was amplified from CP1296 [188] using the primer pair DAM406_*Bam*HI and DAM351_*Apa*I (Annex, table A1). The *cpsJ* DNA fragment and the Janus cassette were digested with restriction enzyme *Bam*HI and ligated with ligase. This *cpsJ*-Janus ligation product was amplified by PCR using primer pair cpsJ_BM47_F1, DAM351_*Apa*I (Annex, table A1). Further this PCR product and DNA fragment cpsP_C_F1_*Apa*I-cpsP_BM54 were digested with restriction enzyme *Apa*I and ligated to obtain the final construct *cpsJ*-Janus-*cpsP* (Fig. 1A). Restriction enzymes and ligase were purchased from New England Biolabs (Frankfurt am Main, Germany).

Transformation of D39SmR was attempted several times with the *cpsJ*-Janus-*cpsP* construct but unfortunately this procedure was not successful. Control PCR's showed either bands of an unexpected size or no band. In a further step, the construct *cpsJ*-Janus-*cpsP* was reconstructed by fusion PCR. The Janus cassette, *cpsJ* and *cpsP* were amplified with overlapping primers (Annex, table A1). After fusion PCR with a mixture of Janus cassette, *cpsJ* and *cpsP* PCR products, the construct was generated. D39 was transformed with this fusion PCR product. Only a few transformants could be selected by 300 µg/ml streptomycin.

4.1.2.4 Construction of the *cpsJ-cpsP* DNA fragment by fusion PCR

The construct *cpsJ-cpsP* (Fig. 1D) was created by fusion PCR. All overlap-primers were designed by using the Lasergene Software (DNASTAR Inc., Madison, Wis). First the genes *cpsJ* and *cpsP* were separately amplified with the forward primers cpsJ_BM47_F1, BM113partBM114_F1 and with reverse primers cpsJ_BM113b1, cpsP_BM54 (Annex, table A1) from the chromosomal DNA of D39 by using Fast Taq DNA polymerase as described above (chapter 4.1.2.3). The two PCR constructs were purified with a QIAquick PCR purification kit (QIAGEN, Basel, Switzerland). The correct size of DNA fragments was confirmed by agarose gel electrophoresis.

Fusion PCR was performed with primers cpsJ_BM47_F1 and cpsP_BM54_b1 (Annex, table A1) by using the following cycling conditions: primary denaturation for 2 min at 95°C, followed by 30 cycles consisting of 94°C for 1 min, 57°C (annealing temperature) for 1 min and 72°C for 2 min (extension time) and then the last cycle for 10 min at 72°C [190]. As template a mixture of the two PCR product *cpsJ* and *cpsP*, both with overlap primers (Annex, table A1), were used. The correct size of the fusion-PCR product *cpsJ-cpsP* was examined by agarose gel electrophoresis and purified with a QIAquick PCR purification kit (QIAGEN, Basel, Switzerland).

4.1.2.5 Sequencing of the fusion PCR product *cpsJ-cpsP*

The fusion-PCR-product *cpsJ-cpsP* was sequenced with an ABI PRISM 310 genetic analyzer (PE Biosystems, Rotkreuz, Switzerland) using a Big Dye terminator cycle sequencing Ready Reaction kit (PE Biosystems). For sequencing the primers cpsJ_end and cpsPstart (Annex, table A1) were used. Amplification was performed

using the following cycling conditions: primary denaturation for 1 min at 96°C, followed by 25 cycles consisting of 96°C for 10 s, 50°C for 5 s and 60°C for 7 min. The DNA sequences were analyzed and aligned by using the Lasergene software (DNASTAR Inc., Madison, Wis.) The *cpsJ-cpsP* construct was compared with original sequences of D39, obtained from GenBank, D39 NC 008593 (BLAST search (blastx) at the National Center for Biotechnology, Information website: http://www.blast.ncbi.nlm.nih.gov/Blast.cgi).

4.1.2.6 Insertion of the *ugd* gene in *lacE* operon of strain D39

The gene *ugd* was amplified by PCR with primers endaad9_ugd_F1 and ugd_B2. All primers are listed in Annex table A1. Primer endaad9_ugd_F1 contained a 25bp sequence that overlaps the 3' end of the *aad9* (spectinomycin resistant cassette), which was kindly provided by M.E. Winkler, Indiana University Bloomington, USA, [191]. *aad9* and its promoter were amplified by PCR with primers spcF1 and spcR1 from plasmid pR412. The product of endaad9_ugd_F1 and ugd_B2 PCR was joined to *aad9* by using fusion PCR (also called SOE: splicing by overlap extension) [190] to generate the *aad9-ugd* cassette. This PCR construct *aad9-ugd* was flanked by 860 bp DNA identical to the regions 5' and 3' of the *lacE* PTS operon (*SPD0423* to *SPD0429*) to target the cassette to this region of the chromosome. The *lacE* 5' region, *lacE(up)* was amplified from D39 genomic DNA by using the primer pair lacEF1-lacER1.

In a separate PCR the *aad9-ugd* cassette was amplified by using primers lacEsp and ugd_B2, creating a fragment that has a sequence overlap with the 3'end of the lacEF1-lacER1 product. Both of these two fragments were fused by using SOE to create *lacE(up)-aad9-ugd*. In a similar manner, the *lacE* downstream region, *lacE(down)*, was PCR amplified by using the primer pair ugd_lacE_F-lacER2 in which the former has a sequence overlap with the 3' end of *ugd*. This piece was fused to the *lacE(up)-aad9-ugd* cassette by using SOE to create the final complementation cassette *lacE(up)-aad9-ugd-lacE(down)* (Fig. 2). The complementation construct completely replaced the coding region of the *lacE* operon and should express *aad9* and *ugd* constitutively from the *aad9* promoter. Strain D39 and D39SmRΔ*cps*::*Janus* were transformed with the complementation cassette, and transformants were selected for by plating on medium containing

300 µg/ml spectinomycin and were confirmed by PCR. Iyer et al. have previously determined that a deletion-insertion in this locus affected neither in vitro growth nor virulence [126].

4.1.2.7 Growth curves in different culture media

Strains were stored at -80°C using Protect bacterial preservers (Technical Service consultants, Heywood, UK). In order to culture the bacteria, they were streaked out on Columbia sheep blood agar (CSBA) plates and incubated for 24 h 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 – 10 colonies in 5 ml brain heart infusion broth (BHI) (Becton Dickinson, le Pont de Claix, France) containing 5% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). The tubes were placed in a 37 °C water bath for 9h. One hundred microliters of the overnight culture was subcultured in 5 ml BHI with 5% FCS and grown to mid-log phase (OD_{600nm} 0.4 – 0.6). After transferring 100 µl of the mid-log phase culture into a tube with 5 ml BHI, Lacks [192] without glucose or Todd Hewitt broth (Becton Dickinson, le Pont de Claix, France) with 5% yeast extract (Oxoid Ltd, Basingstoke, Hamsphire, UK) (THY), each with different additives (Annex, table A8) the OD_{600nm} was measured every hour. If a strain did not reach the OD_{600nm} 0.2, a default value of 450 min was assigned. For each strain one to two independent growth curve experiments were performed on different days.

4.1.3 Results

4.1.3.1 Overview of genetic manipulations

Construction of the single gene *ugd* knockout mutant has not been successful so far. All other genetic manipulations were successful as listed in table 1.

Table 1. Overview of genetic manipulations

DNA constructs/mutated strains	remarks	status
cpsJ-Janus-*cpsP*	Janus cassette between *cpsJ* and *cpsP* instead of *ugd* gene	successful
cpsJ-cpsP	in frame DNA fragment for replacing *ugd* gene	successful
SPD0423-aad9-ugd-SPD0429	spectinomycin cassette and *ugd* gene flanked by genes of *lacE* operon	successful
D39Δ*rpsL+::rpsLstr1*	D39 with resistant streptomycin cassette (D39SmR)	successful
D39SmRΔ*ugd*::Janus	D39SmR with Janus cassette replacing *ugd* gene	unsuccessful
D39SmRΔ*ugd*::*cpsJ-cpsP*	D39SmR single gene *ugd* in frame knockout mutant	unsuccessful
D39SmRΔ*ugd::cpsJ-cpsP*Δ*SPD0423-SPD0429::aad9-ugd*	D39SmR single gene *ugd* in frame knockout mutant with spectinomycin cassette ugd gene in *lacE* operon	unsuccessful
D39Δ*SPD0423-SPD0429::aad9-ugd*	D39 with spectinomycin resistant cassette and *ugd* gene in *lacE* operon	successful
D39Δ*cps*::JanusΔ*SPD0423-SPD0429::aad9-ugd*	D39 with Janus cassette instead of capsule operon and with spectinomycin cassette and *ugd* gene in *lacE* operon	successful

Single gene *ugd* knockout mutant: Repeated attempts were made to transform D39SmR with the *cpsJ*-Janus-*cpsP* construct but unfortunately this procedure was not successful. Transformants were selected by 300 µg/ml streptomycin. Control PCR's showed either a band of the wrong size or no band.

The reconstruction by fusion-PCR yielded the PCR product *cpsJ*-Janus-*cpsP* of correct length of 2150 bp (Fig. 4). The band was cut out and purified before transformation of D39SmR. There were between 1 and 10 transformants per plate, which were confirmed by PCR's (Fig. 5). PCR 1 (Fig. 6) exhibited the correct size

of the construct in D39. PCR 2 (Fig. 6) showed the correct place of *cpsJ*-Janus-*cpsP* upstream of *cpsJ*. Unfortunately the PCR 3 (Fig. 5) which should reveal the correct place of *cpsJ*-Janus-*cpsP* downstream of *cpsP* represented no bands. Further PCR's with other primers to detect this downstream region failed. Additionally, PCR 4 (Fig. 7) exhibited that *ugd* gene was still in D39SmR, located downstream of *cpsJ*. Therefore the construct was obviously not on the right place as shown by the negative result of PCR 3 and the PCR showing that the *ugd* gene was not in D39SmR. Serotyping of transformants confirmed serotype 2.

A recent publication explains why the experiment may have failed [112]. Xayarath et al. described in their experiment that deletion of *cps2K* is detrimental to the cell, and such mutants can be isolated only in the presence of suppressor mutations that reduce or eliminate capsule synthesis. The number of *cps2K* deletion mutants obtained was small (≤1 km-resistant isolate per 10^6 recipients). This result was consistent with the necessity to transform the rare spontaneous mutants that contained *cps2E* or other suppressor mutations that allowed for survivial in the presence of a *csp2K* deletion. Backtransformation of *cps2K* and repair of the *cpsE* point mutation restored the full parental phenotype [112]. These spontaneous suppressor mutations seemed not to occur in our *ugd* knockout mutants and therefore these *ugd* mutants were not able to survive.

Furthermore, in the *cps2K* mutant, constructed by Xayarath et al. no capsule was detectable using a polyclonal antiserum to the type 2 polysaccharide in indirect and competitive-inhibition ELISA's [112]. However the serotype 2 in our transformants could be confirmed by serotyping with specific antisera from the Statens Serum Insitute (Copenhagen, Denmark) which seems to imply the activity of UDP-glucose-dehydrogenase (UGD).

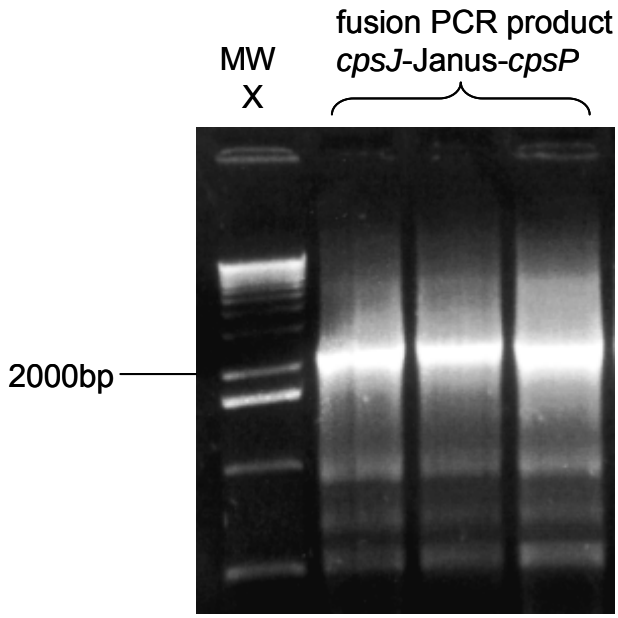


Fig. 4. Fusion PCR product *cpsJ*-Janus-*cpsP* with a length of 2150 bp. MW: Molecular weight marker X.

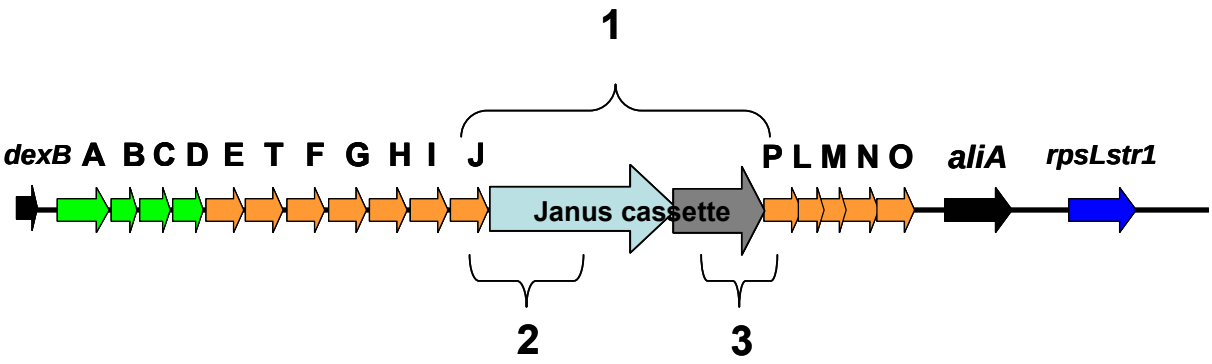


Fig. 5. Determination of correct location and size of the *cpsJ*-Janus-*cpsP* construct in D39SmRΔ*ugd*::Janus by PCR 1 to 3.

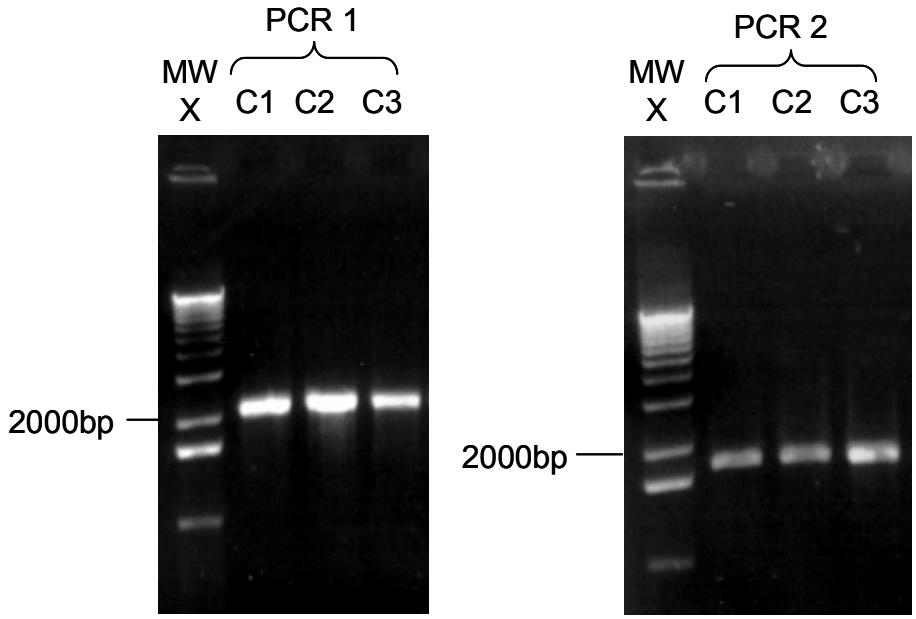


Fig. 6. Gel electrophoresis of control PCR 1 (2200bp) and PCR 2 (1800bp) in D39SmRΔ*ugd*::Janus C1-C3. MW: Molecular weight marker X.

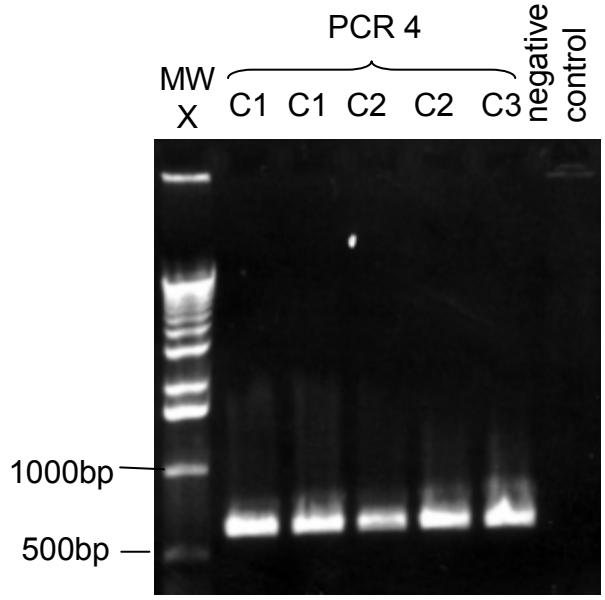


Fig. 7. PCR 4 determines that *ugd* is still in D39SmRΔ*ugd*::Janus C1-C3 (644bp). Negative control with water as template. MW: Molecular weight marker X.

4.1.3.2 *CpsJ-cpsP* DNA fragment

The fusion PCR product *cpsJ-cpsP* contained 1200 bp and was checked by PCR and sequencing reaction. Gel electrophoresis of the PCR product confirmed the correct size (Fig. 8). Sequencing analysis determined the right fusion between the end of *cpsJ* and the beginning of *cpsP*. Therefore the fusion product showed 100 % identity compared to D39, except, the lack of *ugd* gene.

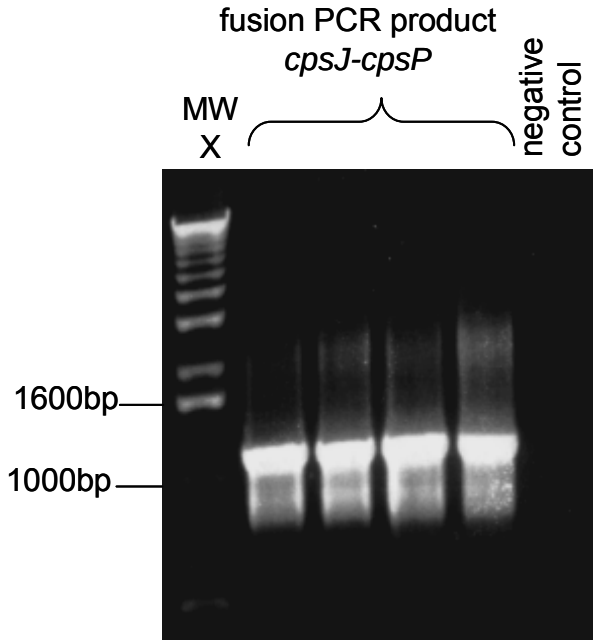


Fig. 8. *CpsJ_cpsP* fusion PCR construct (1200bp). Negative control with water as template. MW: Molecular weight marker X.

4.1.3.3 Insertion of *ugd* gene in *lacE* locus

The complete fusion PCR product *SPD0423-aad9-ugd-SPD0429*, containing 3900 bp, was transformed into D39 and D39SmRΔ*cps*::Janus. Control PCR's were performed from D39 and D39SmRΔ*cps*::Janus (Fig. 9). Gel electrophoresis of PCR I (Fig. 10A) with DNA fragment of 1611bp exhibited the correct place of the construct downstream of *lacE* operon. PCR II (Fig. 10B) (1410bp) showed the correct place of the construct upstream of *lacE* operon and PCR III (Fig. 11A) (455bp) confirmed the right ligation order of the construct. With PCR IV (Fig. 11B) (4140 bp) the correct size of the construct was determined.

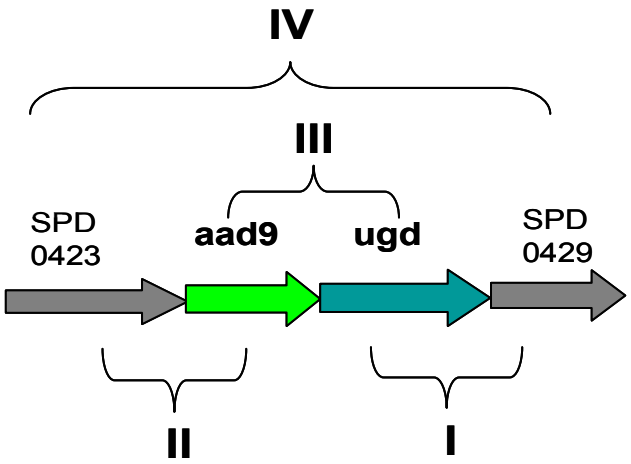


Fig. 9. Confirmation of correct location and size of the *SPD0423-aad9-ugd-SPD0429* PCR construct by PCR's.

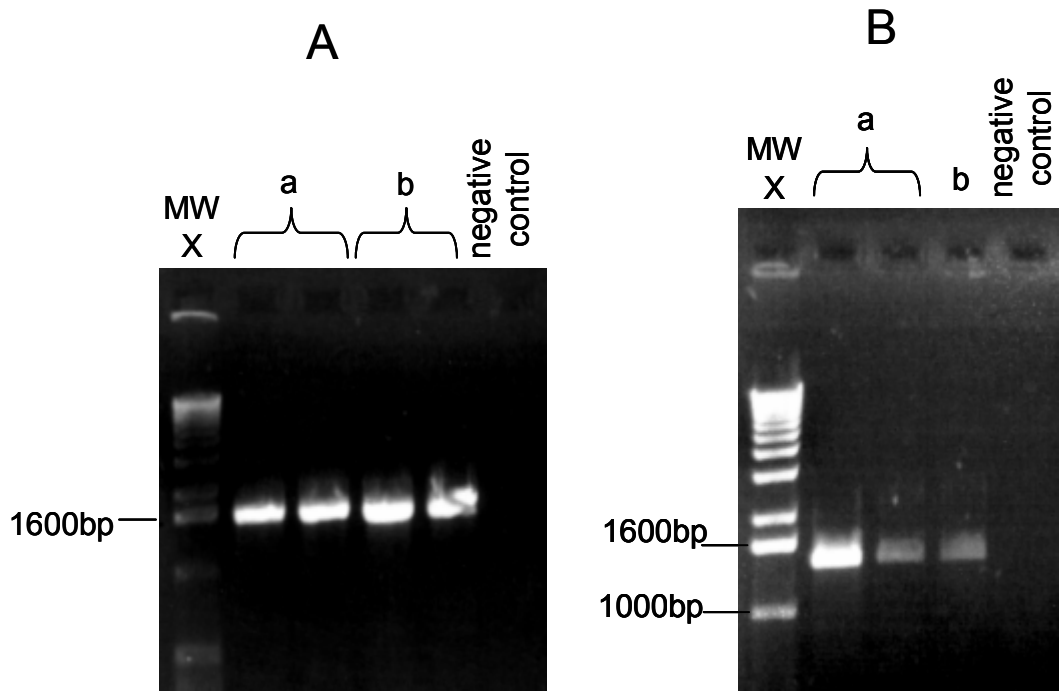


Fig. 10. A) PCR I: confirmation of *ugd* in *lacE* downstream (1611bp). B) PCR II: confirmation of spectinomycin cassette in *lacE* upstream (1410bp). Template a) D39 C1,C2. Template b) D39Δcps::Janus C1. Negative control with water as template. MW: Molecular weight marker X.

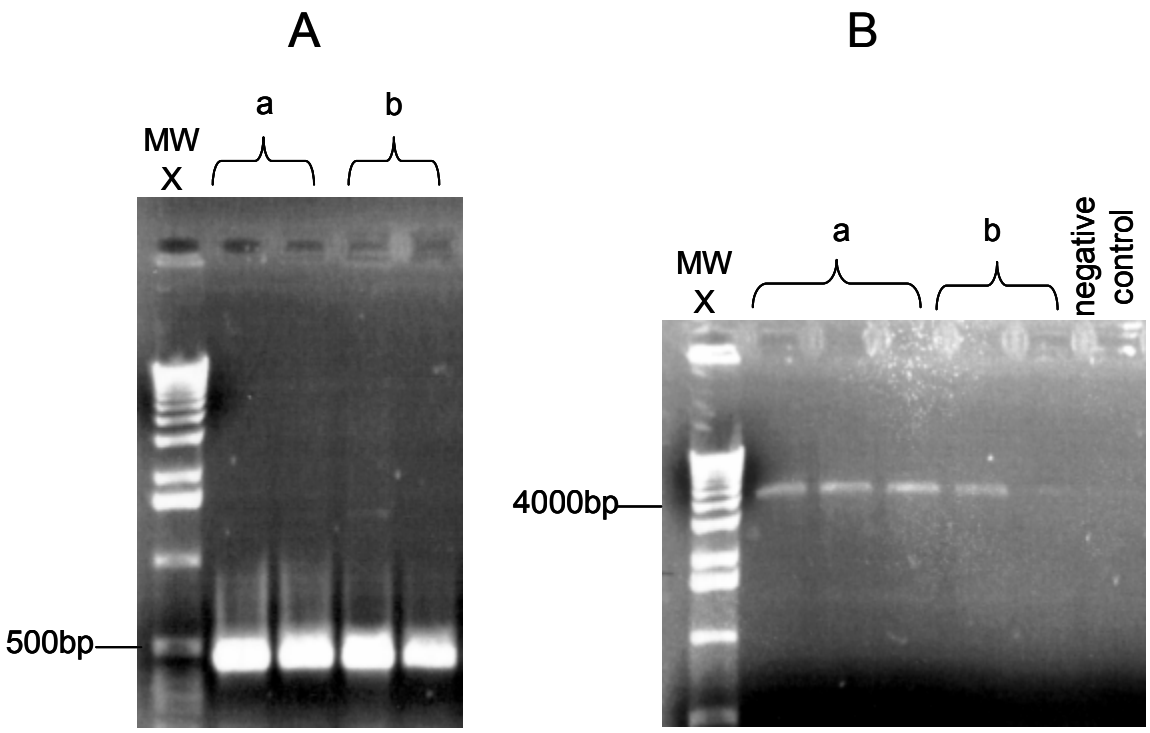


Fig. 11. A) PCR III: confirmation of *aad9-ugd* construct (455bp). B) PCR IV: confirmation of the size and location of *SPD0423-aad9-ugd-SPD0429* (4140bp). Template a) D39 C1, C2. Template b) D39Δcps::Janus C1. Negative control with water as template. MW: Molecular weight marker X.

4.1.3.4 Search for different culture media

To investigate the role of different carbon sources in capsule gene deficient mutants, the literature was searched for different culture media. The aim was to find chemically defined media. The influence of added components such as different sugars or aminoacids during growth curves in a chemically defined medium would be easier to determine than a complex undefined medium. Two chemically defined media were found.

First the media Cden (Annex, table A3) supplemented with choline chloride was described by Tomasz et al. [193].

Second, the CDM medium (Annex, table A4), is a rich medium, which is used for growth of group A streptococci [194]. For pneumococci supplementation of CDM with choline chloride (5 µg/ml), asparagine (50 µg/ml) and sodium pyruvate (250 µg/ml) is recommended [195].

A semisynthetic medium first described by Adams et al. [196] and modified by Lacks et al. [192], (called “Lacks” or “C-medium”) (Annex, table A5) contained the undefined

component caseinhydrolysate. Small modifications of the same medium were described by Lacks et al. or Tomasz et al. [197, 198] (Annex, table A6). C + Y medium is C-medium supplemented with yeast extract.

CAT medium (Annex, table A7) consists mainly of caseinhydrolysate and was described first by Porter et al. [199] and modified by Morrison et al. [200].

Todd Hewitt broth was originally developed for use in the production of streptococcal hemolysin [201] and modified by Updyke et al. [202]. This medium is highly nutritious due to its content of peptones, dextrose and salts. Dextrose stimulates hemolysin production. Sodium phosphate and sodium carbonate provide buffering action to counteract the acidity produced during fermentation of dextrose, thereby protecting hemolysin from inactivation by the acid [203].

Tryptic soy broth (TSB), a soybean-casein digest medium, is a nutritious medium which supports the growth of a wide variety of microorganism, including common aerobic, facultative and anaerobic bacteria and fungi [203, 204]. Enzymatic digest of casein and soybean meal provide aminoacids and other complex nitrogenous substances. Dextrose is an energy source, and sodium chloride maintains the osmotic equilibrium and dibasic potassium phosphate acts as a buffter [205].

Brain heart infusion broth (BHI) is a nutritious, buffered culture medium that contains infusions of brain and heart tissue and peptones to supply protein and other nutrients necessary to support the growth of fastidious and nonfastidious microorganisms. It was first described by Rosenow in 1912 [206] and later modified and described in the National Formulary [207].

4.1.3.5 Growth curves in different culture media

Growth curves with 6 clinical isolates and 6 capsule switch mutants in Cden and CDM were performed. Unfortunately none of these strains reached the OD_{600nm} 0.2 in 450 min. Therefore these media were not used for further experiments.

Growth curves were done in three different media with additional components (Annex, table A8) of the laboratory strain D39, two clinical isolates 204.76 (serotype 1) and B112.30 (serotype 5) and two unencapsulated strains 110.58 and R6. The medium “Lacks” [197] (Annex, table A6) used for this experiment was prepared without sugar. This allowed measurement of the direct influence of each sugar. The lag phase of growth was of particular interest and so the time to reach OD_{600nm} 0.2 was measured (Fig. 12 – 16).

Lacks

Lacks without sugar, without BSA: Only one of five strains, strain R6, showed growth, however with a long lag phase of 296 min.

Lacks without sugar, with BSA: There was no growth of four of five strains. Only strain R6 showed growth, but with a long lag phase (similar to growth in Lack without sugar and without BSA).

Lacks without sugar, with FCS: Only two of four strains revealed growth, but with a long lag phase of 249 to 303 min.

Lacks with sugar: Addition of different sugars (glucose, sucrose, lactose, maltose) significantly shortened the lag phase in three of five strains. The two encpasulated, clinical strains profited more than the laboratory mutant and nonencapsulated strains.

Lacks with sugar, with FCS: In D39 the addition of sugars shortened the lag phase only in combination with FCS. Glucose and sucrose tended to shorten the lag phase (199 and 218 min) more than maltose or lactose (230 and 308 min). The unencapsulated strain 110.58 did not profit for sugar and FCS.

Lacks with sugar, without BSA, with and without FCS: Removal of BSA did not affect growth of the clinical encapsulated strains or of R6 as long as sugars were present. However, in the clinical unencapsulated strain, removal of BSA in the presence of sugar did enhance growth.

Lacks with NAD: Addition of NAD to Lacks with sugar did not change growth.

Tod Hewitt broth (THY)

THY: One of two strains exhibited growth. 110.58 did not grow, whereas D39 showed a long lag phase of 220 min.

THY with NAD: The addition of NAD had no effect on growth.

THY with FCS: The two tested strains profited of FCS, the laboratory strain with shorter lag phases of 131 min and the unencapsulated strain 110.58 with a lag phase of 210 min.

Brain heart infusion broth (BHI)

BHI: All five strains grew in BHI with lag phases between 156 to 175 min, except strain 110.58 showed a long lag phase of 332 min.

BHI with FCS: Addition of FCS shortened the lag phases of all five strains. Strains D39 and R6 revealed a shorter lag phase by 40 min, 204.76 by 48 min and B112.30 by 63 min. The greatest effect of FCS was observed in strain 110.58 with a reduction of the lag phase by 78 min.

BHI with NAD: Supplementation of NAD enhanced the growth of four of five strains. However, the effect was small, the lag phase shortened only by 11 min in B112.30, 15 min in 204.76 and R6 and by 21 min in 110.58. D39 prolonged the lag phase by 16 min.

BHI with $NaHCO_3$: Addition of $NaHCO_3$ prolonged the lag phase of both of the two tested strains D39 and 204.76. The lag phase increased with higher concentrations of $NaHCO_3$.

BHI with MEM: Addition of MEM prolonged the lag phase in the two tested strains compared to growth in BHI alone.

BHI with sugars: Addition of sugars (GlcUA, UDP-Glc, UDP-GlcUA) in the two tested strains showed in strain D39 a shorter lag phase by 11 min and in 204.76 a prolonged lag phase by 17 min compared with BHI alone.

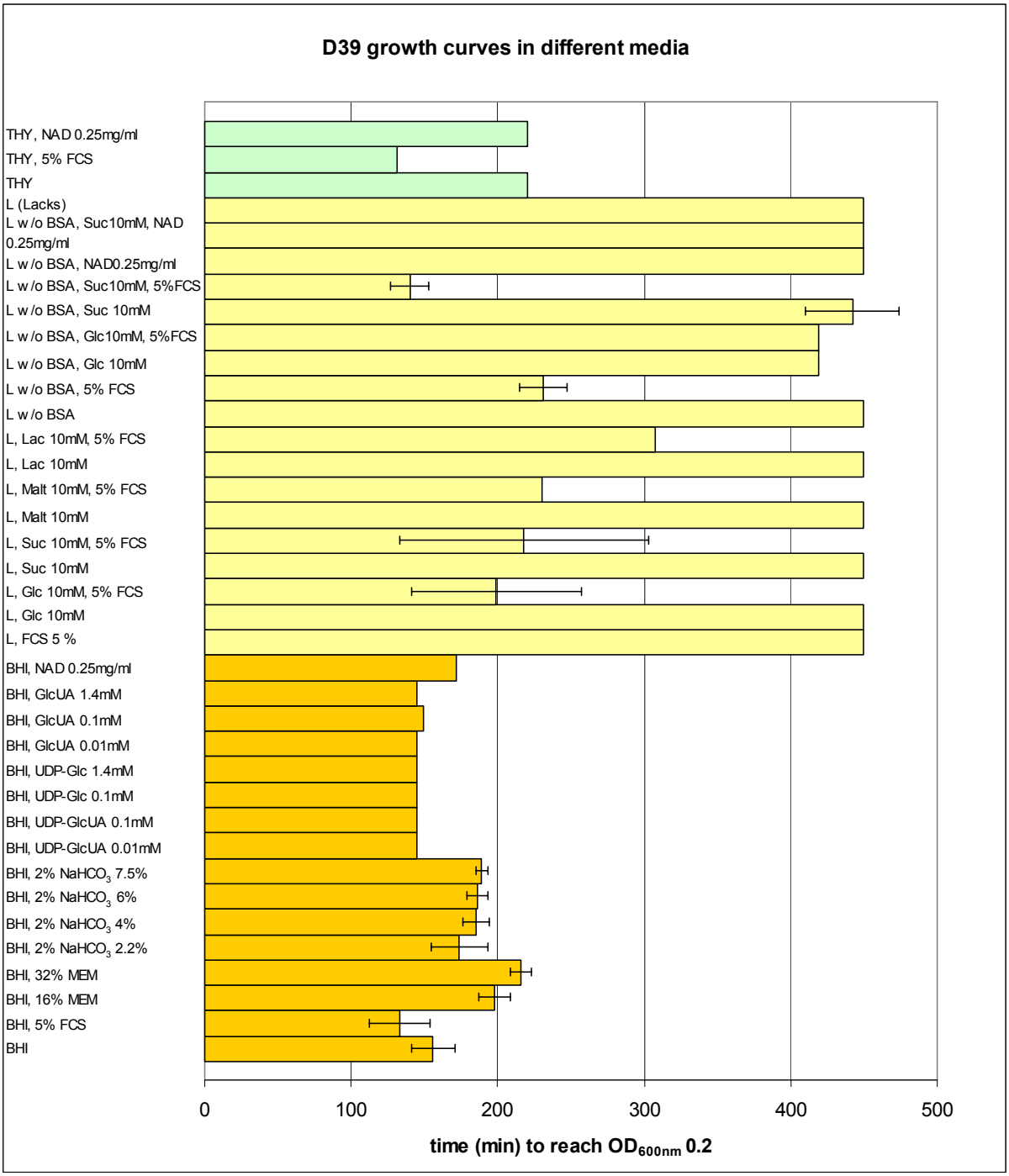


Fig. 12. Time (min) to reach OD_{600nm} 0.2 of D39 (serotype 2) in BHI (orange bars), L: Lacks (yellow bars) and THY (green bars). Growth culture media are described in Annex, table A8. Error bars: standard deviation (SD)

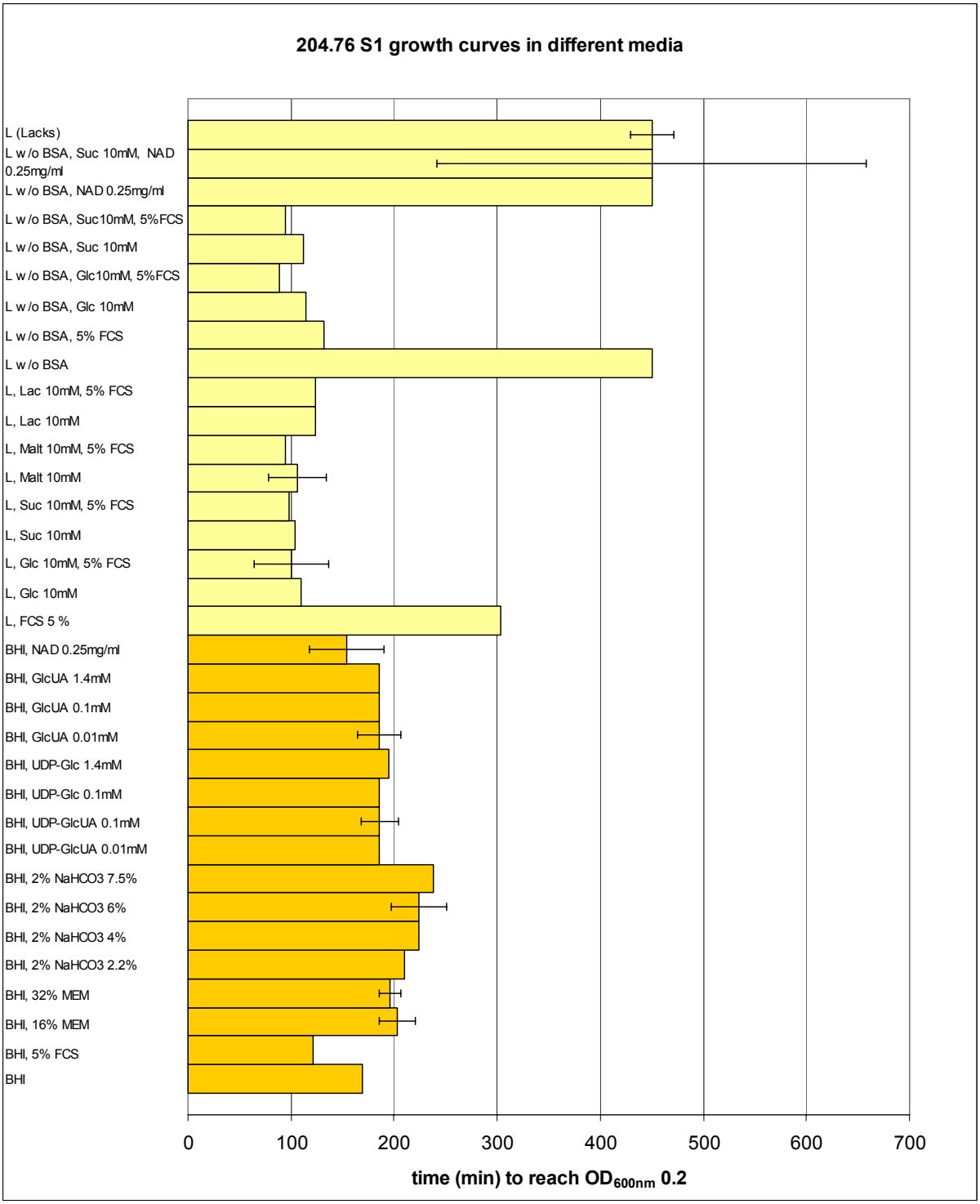


Fig. 13. Time (min) to reach OD_{600nm} 0.2 of 204.76 (serotype 1) in BHI (orange bars), L: Lacks (yellow bars) and THY (green bars). Growth culture media are described in Annex, table A8. Error bars: standard deviation (SD).

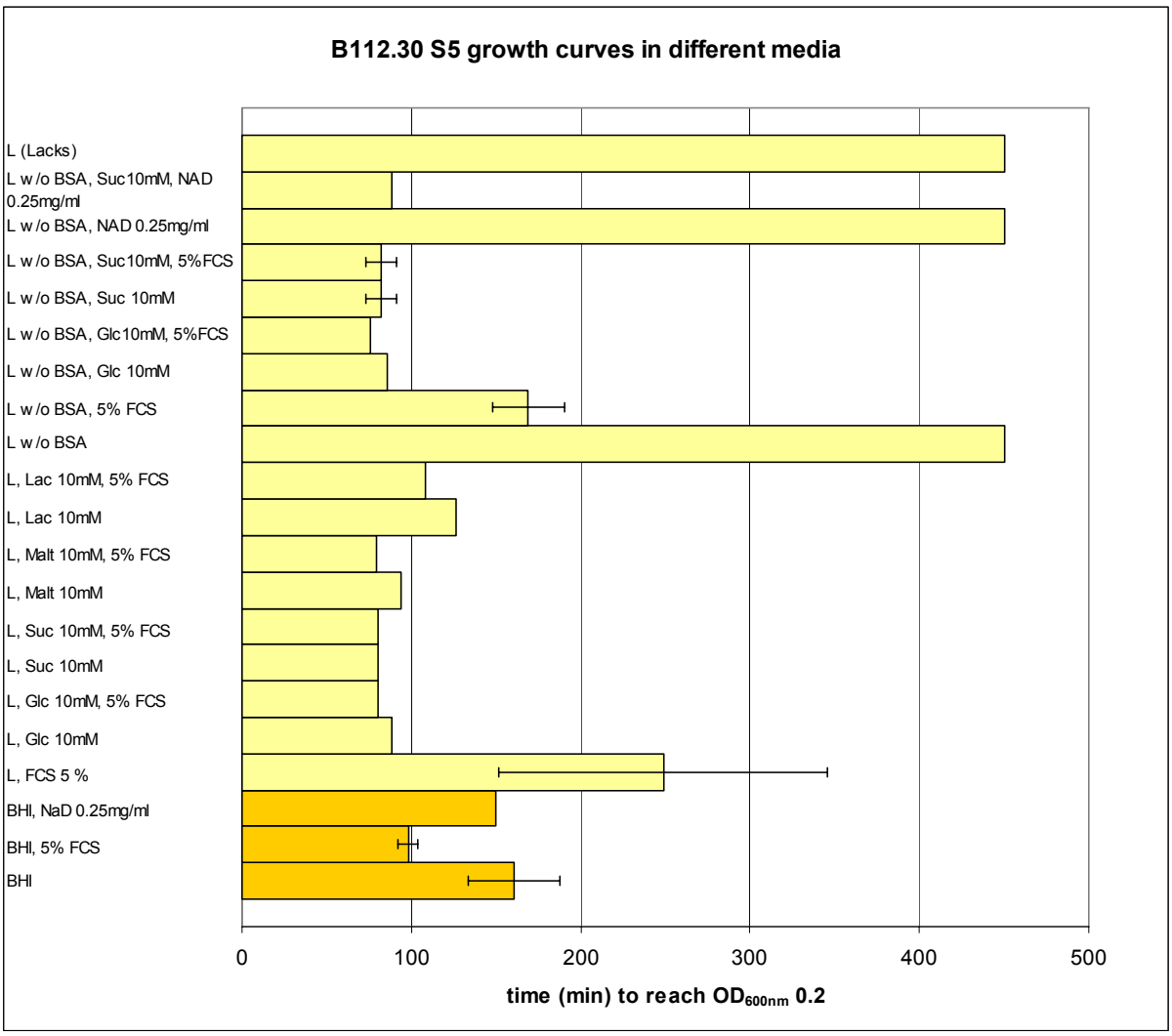


Fig. 14. Time (min) to reach OD_{600nm} 0.2 of B112.30 (serotype 5) in BHI (orange bars), L: Lacks (yellow bars) and THY (green bars). Growth culture media are described in Annex, table A8. Error bars: standard deviation (SD)

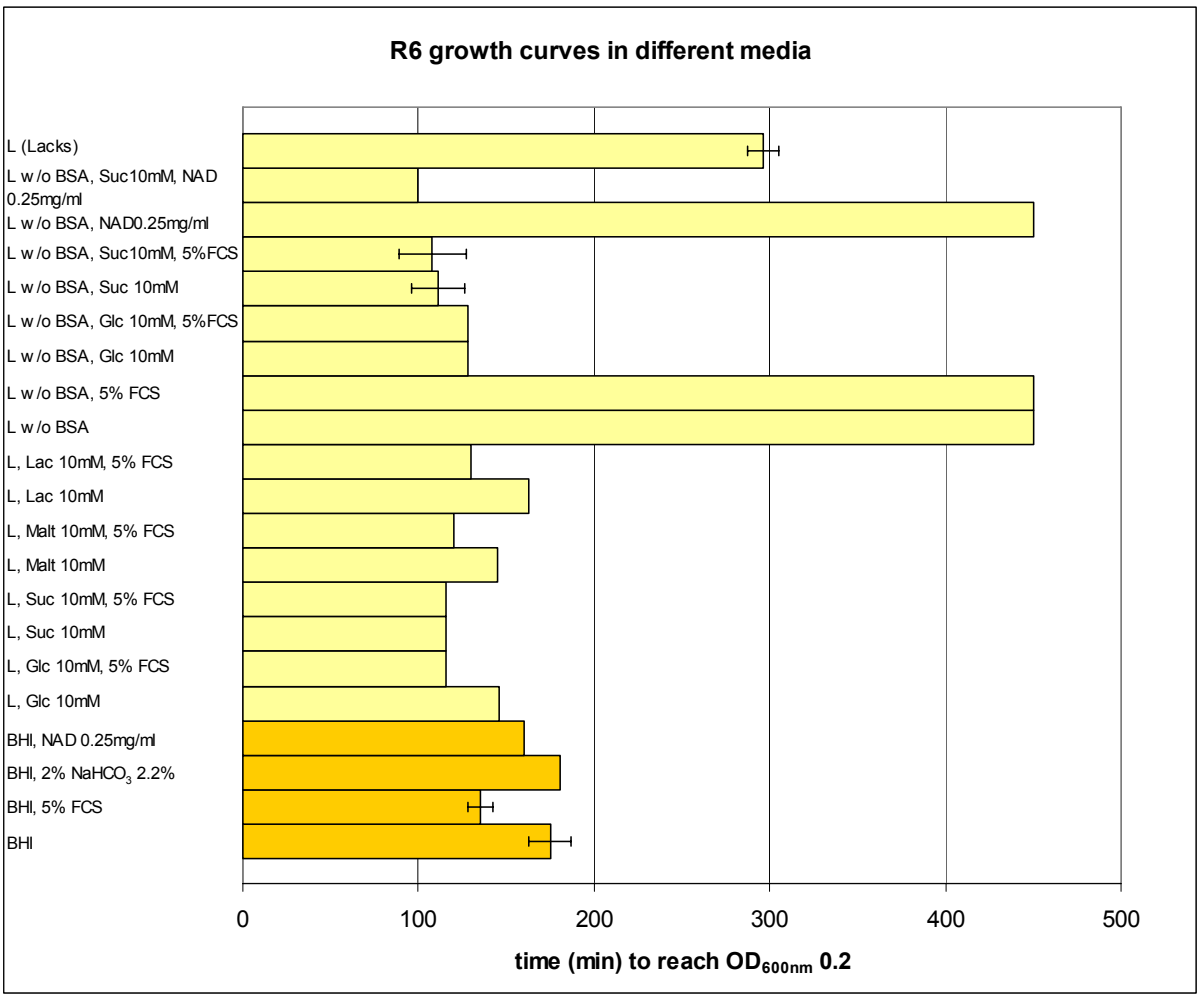


Fig. 15. Time (min) to reach OD_{600nm} 0.2 of R6 (unencapsulated strain) in BHI (orange bars), L: Lacks (yellow bars) and THY (green bars). Growth culture media are described in Annex, table A8. Error bars: standard deviation (SD)

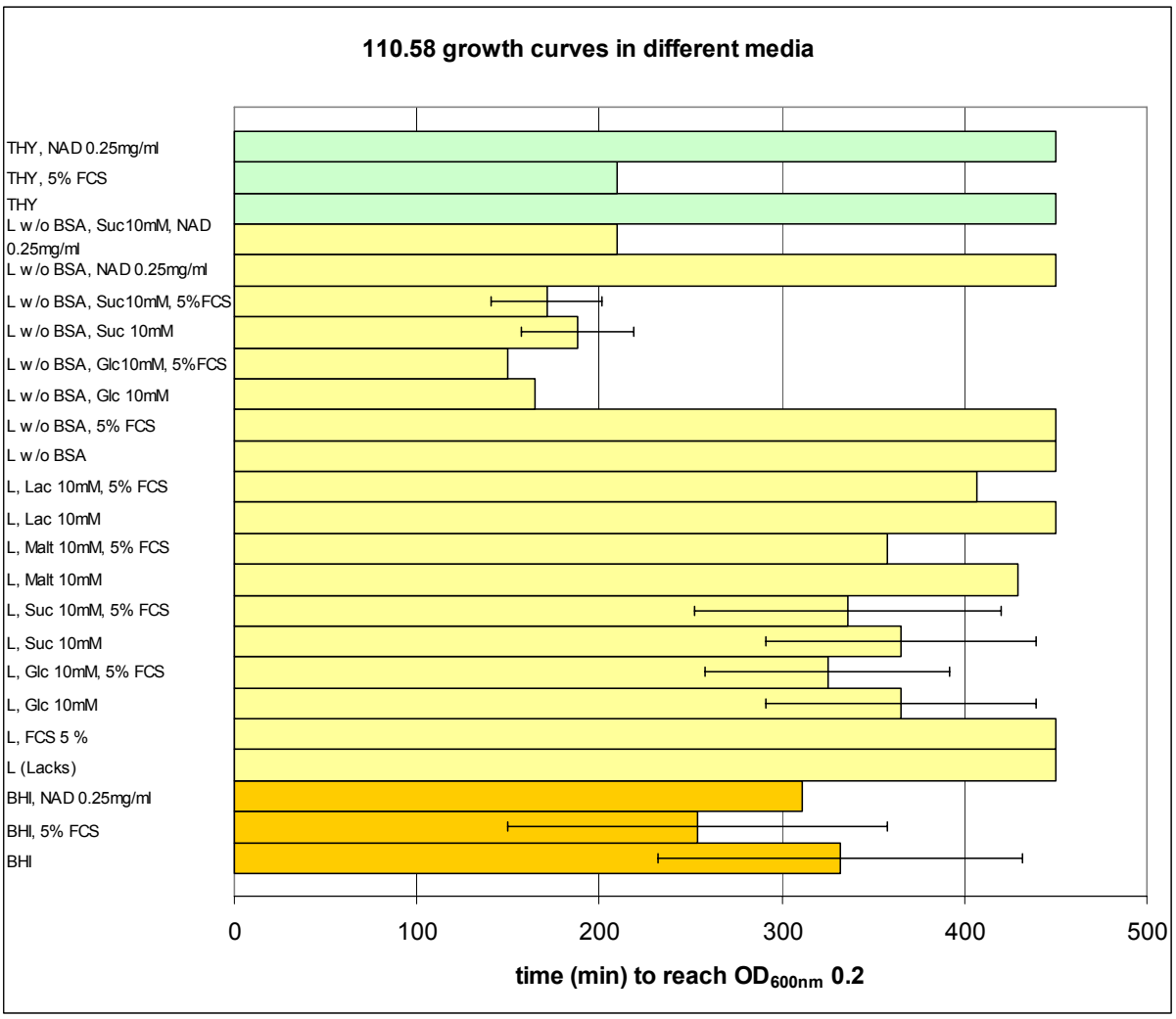


Fig. 16. Time (min) to reach OD_{600nm} 0.2 of 110.58 (unencapsulated strain) in BHI (orange bars), L: Lacks (yellow bars) and THY (green bars). Growth culture media are described in Annex, table A8. Error bars: standard deviation (SD)

4.2 Does capsule switch transform colonization characteristics?

4.2.1 Aim

I tested whether capsule switch between colonizer and invader *S. pneumoniae* serotypes transforms characteristics from an invader phenotype into a colonizer phenotype and vice versa in terms of in vitro growth, in vitro adherence and in vivo colonization in an animal model.

4.2.2 Material and Methods

4.2.2.1 Principle of generating capsule switch mutants

For the construction of capsule switch mutants the bicistronic cassette Janus was used [187, 188]. The Janus cassette in the form of a PCR construct of *dexB*-Janus-*aliA* was kindly provided by K. Trczinski, (Harvard School of Public Health, Boston, USA). Janus is a bicistronic cassette which permits selection both of its acquisition and its loss. Positive selection is based on kanamycin resistance (KanR). Negative selection is based on the resistance to streptomycin.

Streptomycin resistance (SmR) is encoded by a spontaneous mutation in the gene *rpsL*, however, this mutation mediates a recessive resistance phenotype. The Janus cassette contains an intact *rpsL* and strains carrying the Janus cassette appear streptomycin susceptible (SmS), despite the presence of a resistance mutation in *rpsL*. Upon loss of Janus the strain becomes resistant to streptomycin and susceptible to kanamycin (KanS).

Knocking out the capsule operon: The construct *dexB*-Janus-*aliA* was used to transform the laboratory strain D39 (serotype 2) and D39SmR. The transformation was confirmed by PCR. From the strain D39 the PCR fragment with the Janus cassette was amplified for the transformation into clinical isolates.

In a further step a clinical isolate of serotype 5 (strain B101.38; a typical invader) (Fig. 17. 1.) was used as recipient and a clinical isolate of serotype 19F (B201.73; a typical colonizer) served as donor. B101.38 was transformed with the *str1* allele of *rpsL*, which confers streptomycin resistance and contains two silent mutations to prevent gene conversion during later experiments [187]. The different alleles of *rpsL* were all sequenced and compared before choosing *rpsL str1*. B101.38SmR was transformed with Janus cassette under anaerobic conditions, selected by kanamycin

resistance (KanR) and streptomycin susceptibility (SmS) with replica plates (Fig. 17. 2.)

Insertion of another capsule operon: The chromosomal DNA of strain B201.73, serotype 19F (donor) was used to transform into the mutant (B101.38Δ*cps*::Janus) (Fig.17. 3.). Then the new serotype of the recipient strain was determined by the agglutination reaction.

Backcross transformation was performed three times to avoid additional transformation events [188]. This procedure was done by isolating and purifying the chromosomal DNA of the capsule switch mutant and retransforming it back into the mutant with the Janus cassette. The capsule transformants were screened for streptomycin resistance, smooth colony morphology and agglutination reaction. PCR reactions were done of the whole capsule operon and of the junction between the transformed fragment and the capsule flanking regions *dexB* and *aliA*. The region upstream and downstream of the capsule operon was also amplified, digested with restriction enzymes and the pattern was compared with the donor strain.

Additionally, 110 capsule switch mutants (including also capsule exchange within invader strains and capsule exchange within colonizer strains) were generated in the same way (Annex, table A9-A12). Mutants with the Janus cassette are listed in Annex, table A13.

The capsule switch mutant described above was named B101.38cpsB201.73 (Fig. 17. 3.) whereby the first number stands for the recipient strain and the second number for the donor strain. Finally, the capsule switch mutants, mutants with the Janus cassette, and the wildtype strains were further tested for in vitro growth.

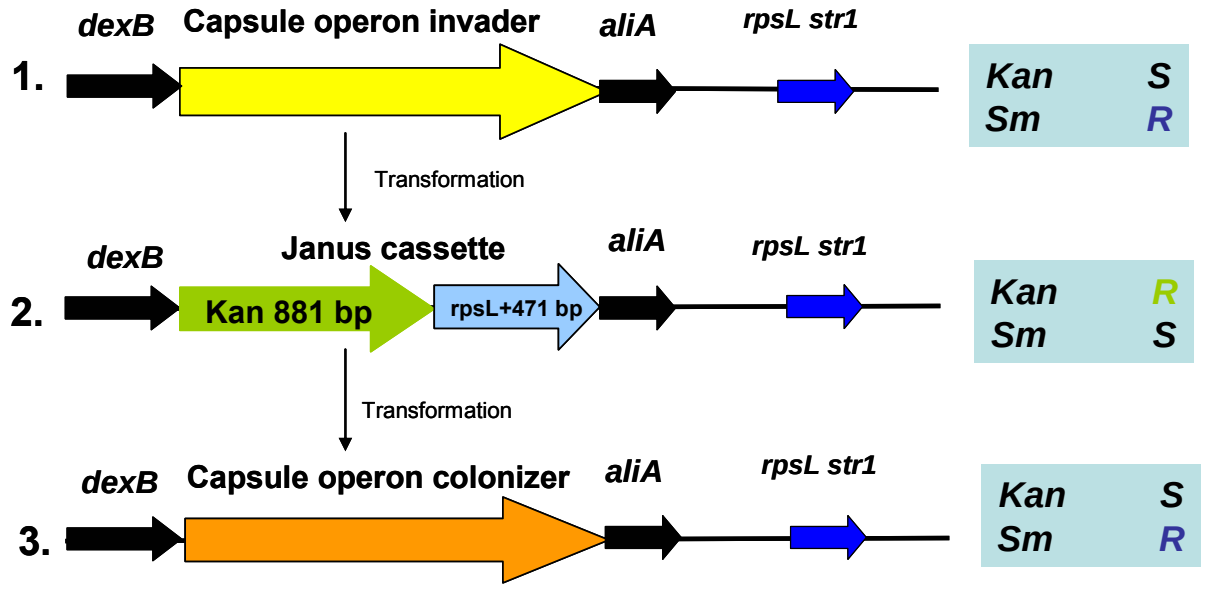


Figure 17: Principle of constructing capsule switch mutants. 1. Original clinical isolate B101.38 (invader) transformed with the *rpsL str1*. 2. B101.38 with the Janus cassette: B101.38Δ*cps*::Janus. 3. Capsule switch mutant consists of a colonizer capsule operon: B101.38cpsB201.73
Kanamycin resistant (KanR), kanamycin susceptible (KanS), streptomycin resistant (SmR), streptomycin susceptible (SmS).

4.2.2.2 Bacterial strains

Clinical isolates of *Streptococcus pneumoniae* were selected from two nationwide surveillance programs collecting nasopharyngeal and invasive isolates [6, 16, 51]. Strains used as recipient and donors respectively included a selection of 12 serotypes known for their enhanced abilitiy to colonize the human nasopharynx (serotypes 6A, 6B, 15, 18C, 19F, 23F) or, in controverse, for low colonization prevalence and high invasive potential (serotypes 1, 4, 5, 7F, 9V, 14) (table 2) [102]. Pneumococcal laboratory strain D39 (serotype 2) was kindly provided by Prof. Jeffrey Weiser (University of Pennsylvania, Philadelphia, USA). All clinical isolates were tested for kanamycin resistance by macrobroth dilution.

Table 2. Strains used in this study. Colonizer serotypes are marked orange and invader serotypes are marked yellow

strain	serotype	RFLP	used as donor/recipient
202.67	1	**15**	donor
B106.79	1	**37**	donor
203.39	4	**17**	donor
B204.27	4	**17**	donor
B101.38	5	**28**	recipient
B203.76	5	**38**	donor
203.24	6A	**11**	recipient/donor
304.78	6A	**11**	recipient/donor
106.66	6B	**3**	recipient/donor
B110.06	6B	**19**	recipient
208.41	7F	**8**	recipient/donor
B109.15	7F	**8**	recipient/donor
B110.04	7F	**8**	recipient
109.74	9V	**1**	donor
201.38	9V	**1**	donor
201.66	9V	**1**	donor
201.12	14	**5**	donor
B101.77	14	**8**	donor
B103.66	14	**1**	recipient/donor
207.31	15C	**1**	donor
307.14	18C	**14**	recipient/donor
B112.27	18C	**14**	donor
B201.61	18C	**16**	recipient/donor
108.34	19F	**39**	donor
B201.73	19F	**40**	recipient/donor
111.46	19F	**4**	donor
202.47	19F	**4**	donor
201.47	19F	**7**	donor
103.57	23F	**11**	recipient/donor
203.29	23F	**11**	recipient/donor

4.2.2.3 Macrobroth dilution for antibiotic susceptibility testing

To check, whether kanamycin selection marker of the Janus cassette can be used, macrobroth dilution was done for kanamycin (Lot 60615, Fluka Biochemika, Buchs, Switzerland) in cation-adjusted Mueller-Hinton broth (Becton Dickinson, le pont de Claix, France), according to the Clinical and Laboratory Standards guidelines [208].

4.2.2.4 Serotyping

For serotyping, the Quellung reaction was used with specific antisera from the Statens Serum Insitute (Copenhagen, Denmark). After the transformation with the Janus cassette the loss of capsule was determined by the rough appearence of

colonies and lack of reaction with any of the pool sera. Successful capsule switch mutants were determined by serotyping for the type of the “donor” capsule.

4.2.2.5 Replica plating

Replica plating was used for selection of successful capsule switch mutants. A filter paper was pressed onto the CSBA agar plate containing 300 µg/ml streptomycin with the transformants and was then transferred to a second CSBA agar plate containing 500 µg/ml kanamycin and a CSBA agar plate without antibiotics. Mutants found on the CSBA plate without antibiotics, but not on the CSBA agar plate with kanamycin showed the correct resistant pattern for capsule switch mutants.

4.2.2.6 Polymerase chain reaction (PCR) to prepare DNA fragments for transformation

All primers were designed by using the Lasergene Software (DNASTAR Inc., Madison, Wis.), (Annex, table A2). The allele *rpsL str1*, which was kindly provided by D. Morrison, University of Illinois, Chicago, USA, was amplified with forward primer DAM350 and reverse Primer DAM351 by using Fast Taq DNA polymerase (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the manufacturer’s instructions. Amplification was performed by using the following cycling conditions: primary denaturation for 5 min at 94°C, followed by 30 cycles consisting of 94°C for 30 s, 50°C (annealing temperature) for 30 s and 72°C for 2 min (extension time) and then the last cycle for 10 min at 72°C.

The Janus cassette was amplified with forward primer dexBstart2 and reverse Primer aliAend2 by using the Expand Long Template PCR system (LRPCR) (Roche Molecular Biochemicals, Rotkreuz, Switzerland). Amplification was performed by using the following cycling conditions: primary denaturation for 2 min at 92°C, followed by 10 cycles consisting of 92°C for 10 s, 65°C for 30 s, and 68°C for 17 min and then 20 cycles in which each extension cycle was prolonged by 20 s. PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Basel, Switzerland).

The size of DNA fragments was examined by agarose gel electrophoresis. The concentration of agarose was selected according to the size of DNA fragments to be analyzed and was between 0.6 to 1%. The DNA molecular weight standards used were Marker II and X (Roche Molecular Biochemicals, Rotkreuz, Switzerland),

depending the DNA fragment weight. DNA concentrations were measured with Perkin Elmer Fluorimeter PIM146, LS-30 (Perkin Elmer AG, Schwerzenbach, Switzerland PIM146, LS-30).

4.2.2.7 Determination of successful transformation

Several PCR's were done to control for the correct size and place of the transformed DNA in mutants with the Janus cassette and in the capsule switch mutants according to the methods described above. For each PCR the annealing temperature was adapted to the melting point of the primers (5 – 10°C below melting points) and the extension time depended on the size of the PCR product. The primers are listed in Annex table 2.

4.2.2.8 Restriction digest and gel electrophoresis

2 – 3 µg PCR product were digested for 3 h with 1 µl of restriction enzyme *Rsa*I for the capsule and *Tsp*509I for the capsule flanking regions. Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany). 1 – 2 µg of the digested DNA were applied to a 0.8 % agarose gel and the gel was run at 80 Volts. Besides the gel electrophoresis, 1 µl of the digestion product was analyzed using the Agilent 2001 Bioanalyzer (Agilent Technologies, Palo Alto, CA) according to the manufacturer's protocol [209].

4.2.2.9 Sequencing

The different *rpsL* alleles (*rpsL+, rpsLstr41, rpsLstr1*) were sequenced with an ABI PRISM 310 genetic analyzer (PE Biosystems, Rotkreuz, Switzerland) by using a Big Dye terminator cycle sequencing Ready Reaction kit (PE Biosystems, Rotkreuz, Switzerland). The sequencing primers used were DAM350, DAM351, rpsLmitteF1, and rpsLmitteB1 (Annex, table A2). Amplification was performed using the following cycling conditions: primary denaturation for 1 min at 96°C, followed by 25 cycles consisting of 96°C for 10 s, 50°C for 5 s and 60°C for 7 min.

The DNA sequences were analyzed and aligned by using the Lasergene software (DNASTAR Inc., Madison, Wis.) The *rpsL* alleles were compared by performing a translated BLAST search (blastx) at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

4.2.2.10 Extraction of chromosomal DNA

Chromosomal DNA was obtained from the donor strains for transformation into the recipient strain (containing Janus cassette), or from the capsule switch mutants for the backcross transformation. The bacteria lawn from two CSBA plates were resuspended in TE buffer (10mM Tris-HCL, 1mM EDTA [pH 8.0]). The bacteria was lysed by the addition of the GES reagent (guanidium isothiocyanate, EDTA, sarcosyl). Ammonium acetate was added to the solution, followed by 10 min of incubation on ice. Chlorofom-isoamyl alcohol was added to extract the proteins, and then the DNA was precipitated with isopropanol and washed with 70% ethanol. The DNA was resuspended in TE buffer and stored at -20°C.

4.2.2.11 Transformation

Transformation procedures were performed for all transformations according to the following protocol. Competent recipient strains were prepared by inoculating BHI with 5% FCS with 3 – 10 colonies and grown overnight. A fresh culture was started in the morning by diluting the overnight culture 1:100 in fresh BHI with 5% FCS and was grown to an OD_{600nm} of 0.15 by using a Perkin-Elmer Lambda-2 spectrometer (Perkin-Elmer AG, Schwerzenbach, Switzerland). An aliquot of the culture was diluted 1:20 in TSB competence medium, pH 8.0 [210] prewarmed at 30°C and incubated for 15 min. Competence stimulating peptide (Neosystem, Strasbourg, France) was added to a final concentration of 200 ng/ml, and the culture was allowed to incubate for 30 min at 30°C.

A total of 1 µg of DNA consisting of the *rpsL str1* DNA fragment, Janus cassette, or 2 µg of the chromosomal DNA of the donor strain and of the capsule switch mutant for backcross transformation, was added to the culture, which was allowed to incubate for 40 min at 30°C and then for 90 min at 37°C. Aliquots of the cultures were then spread onto CSBA plates containing 300 µg/ml streptomycin or 500 µg/ml kanamycin. The plates were incubated for 24 h prior to subculture of single colonies on CSBA plates. After serotyping, successful transformants were stored for further evaluation at -80°C using Protect bacterial preservers (Technical Service consultants, Heywood, UK). In parallel, serial dilutions of the same culture were spread onto CSBA plates to determine the total cell count.

4.2.2.12 Growth curves of capsule switch mutants, mutants with the Janus cassette and their original clinical isolates

In order to culture the bacteria, they were streaked out on Columbia sheep blood agar (CSBA) plates and incubated for 24 h 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 – 10 colonies in 5 ml brain heart infusion broth (BHI) (Becton Dickinson, le Pont de Claix, France) containing 5% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany). The tubes were placed in a 37°C water bath for 9 h. One hundred microliters of the overnight culture was subcultured in 5 ml BHI with 5% FCS and grown to mid-log phase (OD_{600nm} 0.4 – 0.6). After transferring 100 µl of the mid-log phase culture into a tube with 5 ml BBL™ Trypticase™ (TSB) (Lot 6033222, Becton Dickinson, Germany) with 0.3% yeast extract (Oxoid Ltd, Basingstoke, Hampshire) and a tube with only TSB, the OD_{600nm} was measured every hour. For each strain at least three independent growth curve experiments were performed on different days.

4.2.2.13 Statistical analyses

Statistical analyses were done in StatView® version 5.0 (SAS Institute Inc., Cary, NC, USA). 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 or ANOVA respectively. A cutoff *P* value of ≤0.05 (two tailed) was used for all statistical analyses.

4.2.3 Results

4.2.3.1 MIC of clinical isolates

Pneumococcal strains which were used as recipient strains for the transformation with Janus cassette and D39Δ*cps*::Janus were tested for kanamycin susceptibility by macrobroth dilution. All strains tested exhibited a MIC value <500 µg/ml, which is necessary for selection for successful transformants. D39Δ*cps*::Janus showed a MIC >1000 µg/ml proving that the kanamycin resistance cassette is functional (table 3).

Table 3. MIC to kanamycin determinded by macrobroth dilution

Strain	Serotype	MIC kanamycin µg/ml
D39	2	<50
D39Δ*cps*::Janus*	not typable	>1000
103.57	23F	100
304.78	6A	<50
203.24	6A	100
106.66	6B	100
208.41	7F	<50
B109.15	7F	200
B110.04	7F	<50
307.14	18C	100
B201.61	18C	100
B101.38	5	<50
B103.66	14	<50
B201.73	19F	200

D39Δ*cps*::Janus* : contain Janus cassette instead of capsule

4.2.3.2 PCR to prepare allele *rpsL str1* and the Janus cassette

The allele *rpsL str1* and Janus cassette were amplified by PCR and Long range PCR, respectively, and purified with a QIAquick PCR purification kit (QIAGEN, Basel, Switzerland) for transformation of D39 and clinical isolates. Gel electrophoresis showed the correct size of PCR products *rpsL str1* with 451 bp and *dex*-Janus-*aliA* with 5493 bp (Fig. 18).

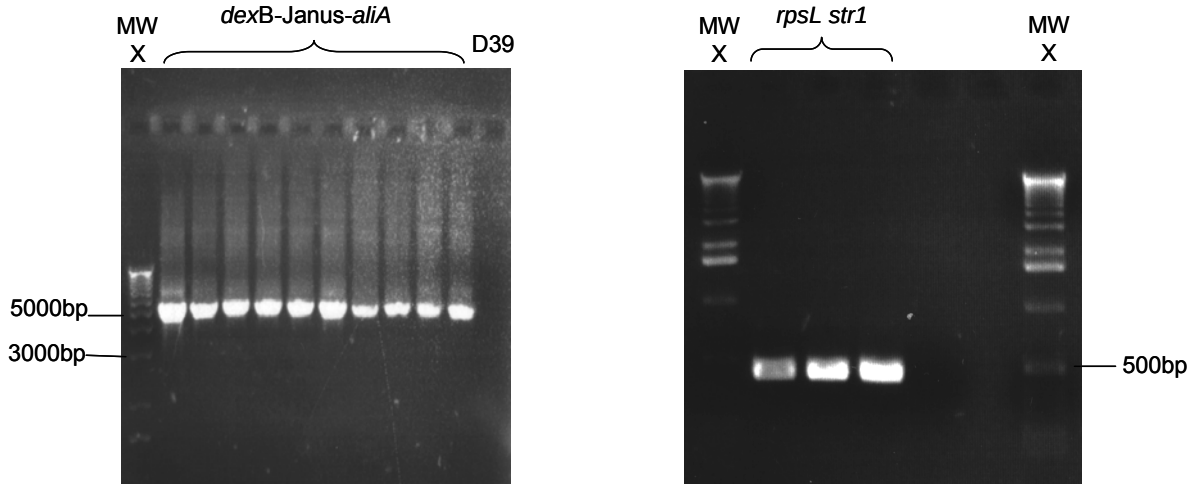


Fig. 18. PCR products *dexB*-Janus-*aliA* (5493bp) and *rpsL str1* (451bp). D39 as negative control. MW: Molecular weight marker X.

4.2.3.3 To avoid gene conversion by using different *rpsL* alleles

By sequencing *rpsL* genes of *S. pneumoniae,* two different mutated *rpsL* alleles, *str1* and *str41* were compared with the published wild type sequence *rpsL*+ (Z15120) [187]. The recessive *str41* mutation correspond to a single base substitution (A to C transversion) converting Lys56 (AAA) to Thr (ACA). The allele *str1* confers the same K56T substitution in protein S12 of the small ribosomal subunit but also carries two silent transitions (GC to AT) at positions 150 and 405 in the *rpsL* gene.

After transformations, streptomycin resistant clones occurred from gene conversion and represented false positive results. During this gene conversion the *rpsL41* allele had replaced the Janus cassette linked *rpsL*+ copy. Obviously the *str41* allele mismatch was not efficiently recognized by HexA, a mismatch repair system [211]. To avoid gene conversion we used *rpsL str1* allele containing two silent mutations which are recognized by HexA, as recommended by Sung et al. [187]. Therefore the frequency of gene conversion was reduced 18 fold.

4.2.3.4 Confirmation of Janus and capsule locus transformation

Polymerase chain reactions were performed to assess mutants with the Janus cassette and capsule switch mutants (Fig. 19). In mutants with the Janus cassette the correct location of the Janus cassette was determined by PCR I (Fig. 20A) and PCR IV (Fig. 21A). The length of Janus cassette was tested with PCR II (Fig. 20B)

and III. To ensure that no additional insertions occurred during transformation PCR VIII was done. In capsule switch mutants the location of the new capsule was determined by PCR V (Fig. 21B) and VI. The length of the new capsule was tested by PCR VII. All mutants showed correct size and location of inserted constructs.

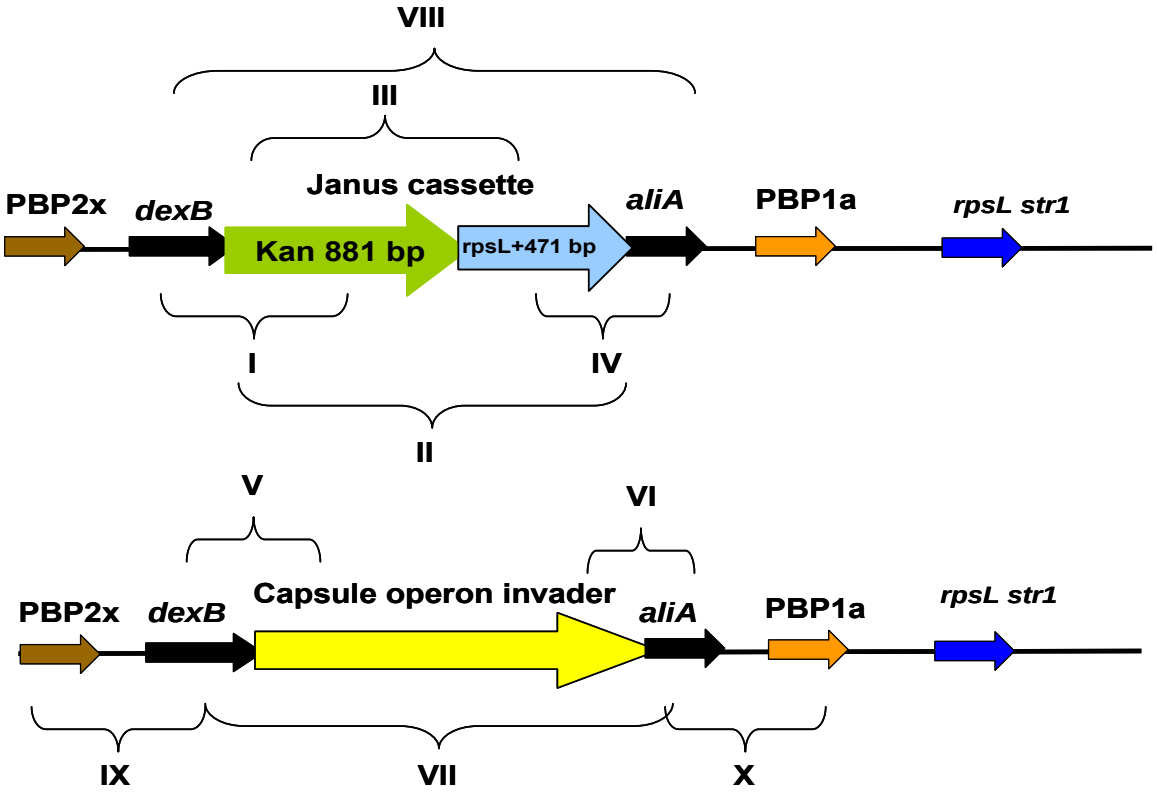


Fig. 19. Overview of control PCR's of mutant with Janus cassette and capsule switch mutant.

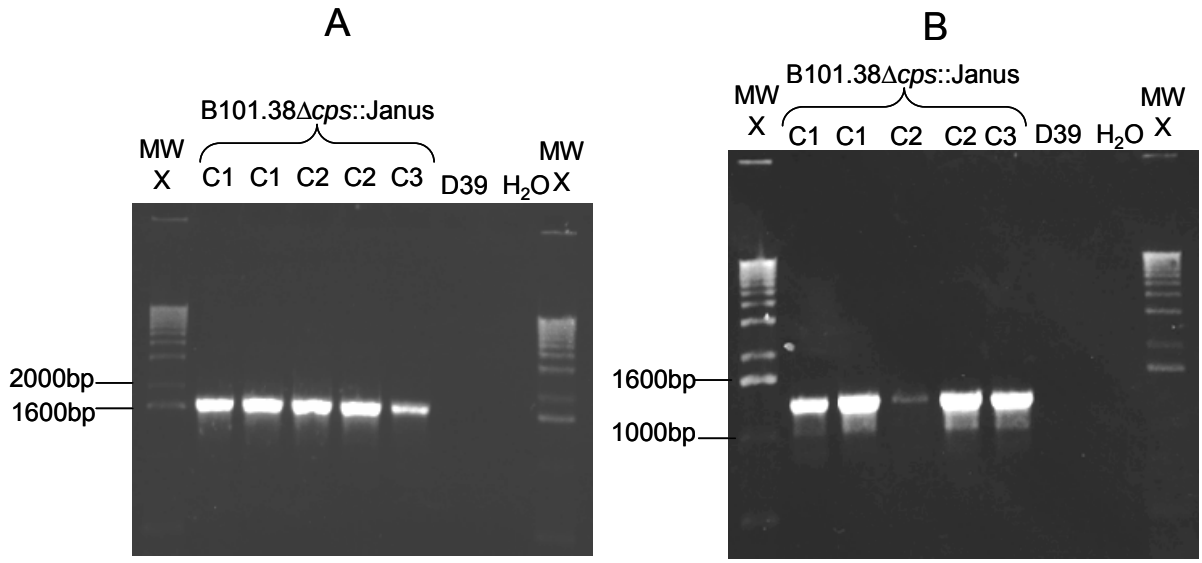


Fig. 20. A) PCR I: confirmation of correct location of the Janus cassette upstream (1700 bp). B) PCR II: confirmation of correct length of the Janus cassette (1400bp). Template: B101.38Δ*cps*::Janus C1-C3 (clinical isolate with the Janus cassette), D39 as negative control for the Janus cassette and H_2O as negative control. MW: Molecular weight marker X.

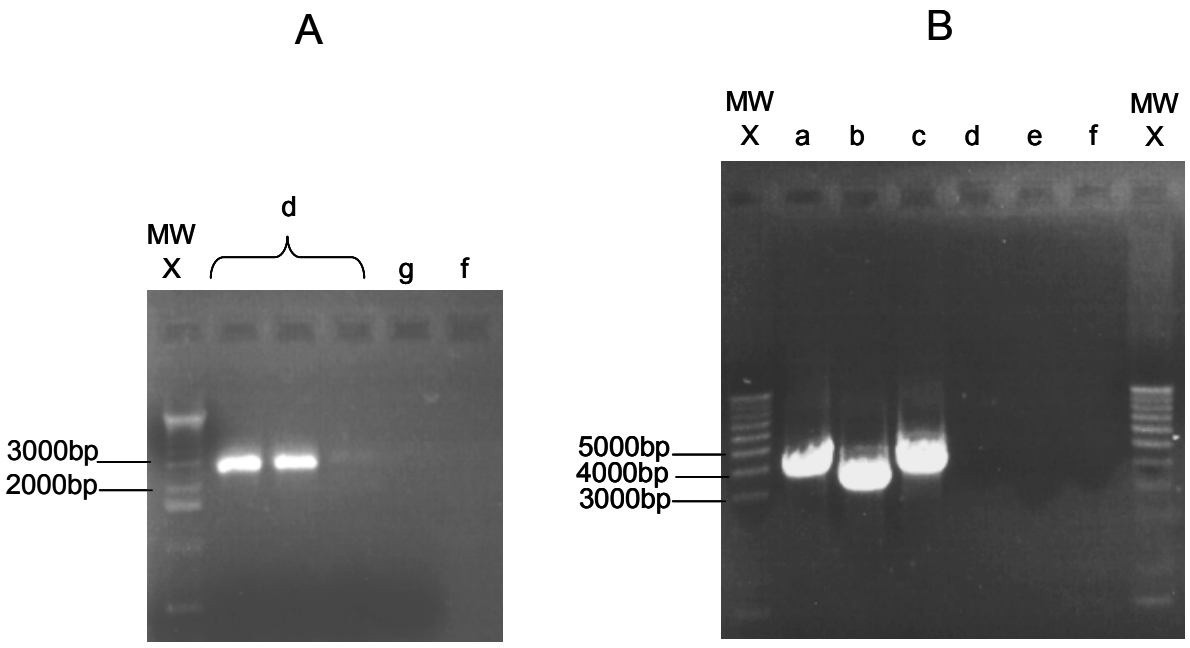


Fig. 21. A) PCR IV: confirmation of correct location of the Janus cassette downstream (2920 bp). B) PCR V: confirmation of the correct location of the new capsule upstream. Template: a) B101.38cspB201.73 (capsule switch mutant), b) B101.38 and c)B201.73, both clinical isolates, d) B101.38Δ*cps*::Janus (clinical isolate with the Janus cassette, e) D39Δ*cps*::Janus, f) H_2O as negative control, g) D39 wt as a negative control. MW: Molecular weight marker X.

4.2.3.5 Analysis of flanking regions in transformants to confirm correct insertion

RFLP analysis of regions flanking the capsule locus revealed different patterns among the analyzed strains B101.38cpsB201.73, B101.38 and B201.73 (Fig. 22). Whereas Tsp509I fingerprints of the *aliA-pbp1a* showed homology between capsule switch mutant (B101.38cpsB201.73), donor (B201.73) and recipient (B101.38) (PCR product X of 8.6 kb, Fig 19, 22.), different fragments were observed for *pbp2x-dexB* (PCR product IX of 9 kb, Fig. 19, 22). *The pbp2x-dexB* region of the capsule switch mutant and the recipient strain match, but are different from that of the donor strain. This result suggested that only the capsule locus was transformed without additional fragments of the flanking region. As expected the *Rsa*I fingerprint of the capsule locus (PCR product VII of 22 kb, Fig. 19, 23) from capsule switch mutant and the donor strain matched, whereas the original recipient capsule locus was different (analyzed by using Agilent 2001 Bioanalyzer). Therefore the capsule replacement was successful.

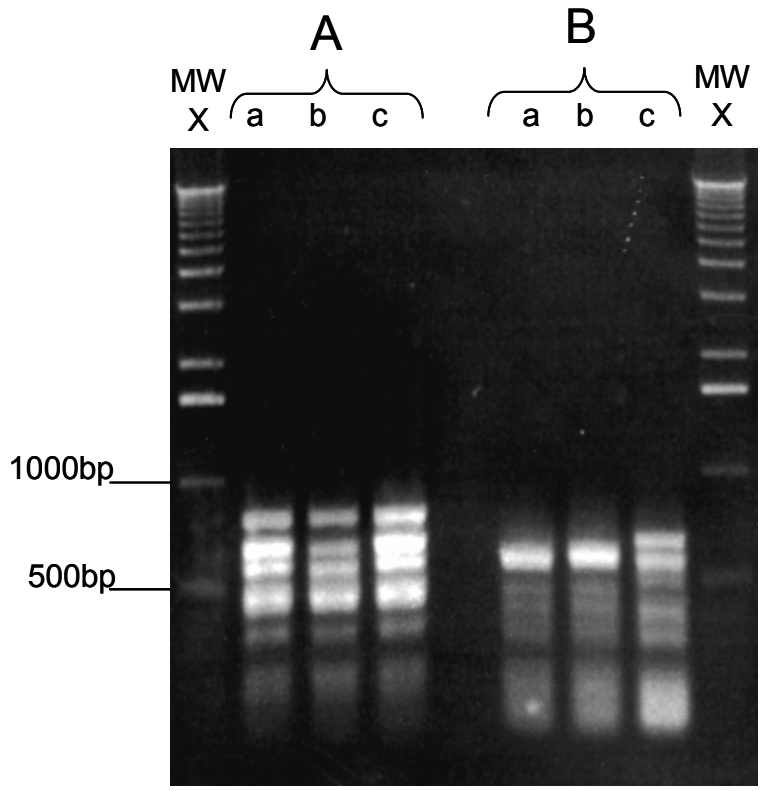


Fig. 22. A) RFLP of the flanking region downstream of the capsule (*aliA-pbp1a*) of PCR X in Fig. 19. B) RFLP of the flanking region upstream of the capsule operon (*pbp2x-dexB*) of PCR IX in Fig. 19. Template: a) B101.38cspB201.73 (cps switch mutant), b) B101.38 and c) B201.73, both clinical isolates.

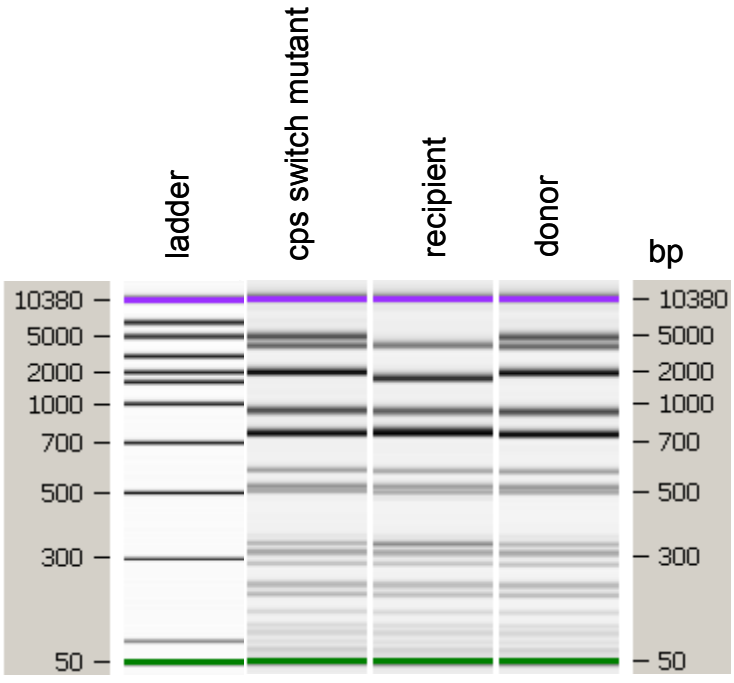


Fig. 23. RFLP of the capsule locus of capsule switch mutant B101.38cpsB201.73 with the recipient strain (B101.38) and the donor strain (B201.73).

4.2.3.6 Backcross transformations to ensure isogenic background

Since chromosomal DNA was used to construct capsule switch mutants, there was a risk that capsular transformants had acquired other, unlinked genetic material from the donor strain. To avoid these additional transformations capsule switch mutants were transformed back into the recipient strain with the Janus cassette. This procedure was performed three times. Trzcinski et al. reasoned that among capsular transformants, the probability that any given locus was cotransformed was less than 1/100, and that if each backcross transformation independently reduced the probability of cotransformation by a factor of at least 100, then the three times backcross transformants should have a probability of less than 10^{-8} of carrying any given gene from the donors apart from the capsule region [188]. In our experiment backcross transformations were not done with all capsule switch mutants. We observed a decreasing transformation rate during backcross transformations. Further we wanted to avoid adaptation of capsule gene expression to growth medium during several transformation steps.

4.2.3.7 Transformability varies between colonizer and invader

Transformation experiments showed that the ability to take up DNA varies between different serotypes. A total of 12 approaches were done to transform the *rpsL+str1* allele and the Janus cassette into two strains of serotype 1 and two strains of serotype 4. Despite the use of different transformation competence media, different competence stimulating peptides and addition of $CaCl_2$, transformation was not successful. Overall eight attempts were also necessary to transform the *rpsL+str1* allele and the Janus cassette into one strain of serotype 15 and two strains of 9V. However, these serotypes, 15 and 9V, were not used as recipients because of limiting the number of capsule switch mutants.

Of 27 transformation experiments between invader serotypes, the recipients were able to take up the new capsule in 17 (63%) experiments (table 4). For 34 transformation attempts between colonizers the success rate was 52.9% (n 18). From a total of 76 invader (recipient) – colonizer (donor) transformations, 24 recipients took up a new capsule (31.6%), and from a total of 79 colonizer (recipient) – invader (donor) transformations, 30 transformations (38%) were successful. The comparison of the transformation rate reached when transforming within categories of invaders or colonizers statistical significance (p=0.004). Backtransformations

(transformation of the capsule of the original strain) showed a high success rate of 100% for colonizer strains and was lower (50 %) for invader strains, although the number of experiments performed was rather small (table 4).

Table 4. Transformability between colonizer and invader, and backtransformations

recipient-donor	experiments performed (n)	successful (n)	successful (%)	p-value
invader-invader	27	17	63.0	
colonizer-colonizer	34	18	52.9	
invader-colonizer	76	24	31.6	0.004*
colonizer-invader	79	30	38.0	
backtransformation invader	6	3	50.0	
backtransformation colonizer	7	7	100.0	

**P*-value compares the transformation efficacy between the following two groups: invader-invader/colonizer-colonizer taken together versus invader-colonizer/colonizer-invader.

4.2.3.8 Transformability varies between different capsule operon sizes

We analyzed whether the transformation rate correlated with the size of the transformed capsule (table 6). The capsule size of each serotype is listed in table 5. The average size of all used colonizer capsules was 19626 bp and of the invader capsule 21331bp.

Table 5. Sizes of capsule operons in basepairs (bp)

Serotype	bp
7F	24127
23F	22330
1	22182
18C	21819
4	20936
9V	20856
5	19969
14	19918
19F	19798
15	18626
6A	17677
6B	17506

Our results showed that colonizers accepted more often capsules of bigger sizes than their own, especially when the capsule came from a colonizer (72.4%) (table 6). Smaller colonizer capsule were accepted with a rate of 26.3%. Colonizer recipients

showed a transformation efficacy of 33.3% for a bigger invader capsule compared to 13.6% for a smaller invader capsule.

Invaders tended to prefer capsules that were smaller than their own, but especially when they came from an invader (70.0%), compared to smaller colonizer capsule 50.8% (statistically not significant). Bigger capsule as donors were taken up of invader with a transformation efficacy of 43.8% for invader and 25.0% for colonizer.

Table 6. Transformability between different capsule operon sizes.

capsule sizes of donor and recipients	experiments performed (n)	successful (n)	successful (%)	p-value
donor invader > recipient invader	16	7	43.8	
donor invader < recipient invader	10	7	70.0	0.2
donor colonizer > recipient invader	16	4	25.0	
donor colonizer < recipient invader	61	31	50.8	0.06
donor invader > recipient colonizer	69	23	33.3	
donor invader < recipient colonizer	22	3	13.6	0.07
donor colonizer > recipient colonizer	29	21	72.4	
donor colonizer < recipient colonizer	19	5	26.3	0.004
overall donor and recipients				
donor capsule > recipient capsule	130	55	42.3	
donor capsule < recipient capsule	112	46	51.5	

4.2.3.9 Effect of capsule switch mutants on growth

First growth curves with 13 capsule switch mutants were performed in BHI without FCS, BHI with 5% FCS and in TSB with 0.3% yeast extract. Time to reach OD_{600nm} 0.2 showed higher differences between capsule switch mutant, recipient and donor in TSB with yeast extract than in BHI with FCS (Annex, Fig. A1, A2). We believed that BHI with FCS was to rich to demonstrate metabolic deficits between capsule switch mutants, recipients and donors. Growth curves in BHI without FCS showed in 6 of the 13 tested strains prolonged lag phases (>250 min) so that we worried, that not all strains would even grow in BHI. TSB with yeast extract differentiated best the lag phases between capsule switch mutants and their original clinical isolates. Therefore we decided to perfom growth curves of 110 capsule switch mutants with their original clinical isolate in TSB with yeast extract (Fig. 24 – 26), (Annex, Fig. A3 – A13). The phylogenetic background of recipient and donor of the capsule switch mutants were clonal in 14 of the 110 capsule switch mutants, as determined by restriction fragment length polymorphism.

As previously shown [145] deletion of the capsule operon by insertion of the Janus cassette did prolong the lag phase (26 min, SE 16.47), (Fig 26). Also, transformation of a new foreign capsule operon hindered growth by prolonging the lag phase. However, the difference between the lag phase of the wild-type recipient strain and the capsule switch mutant (extra delay) correlated with the colonization/invader characteristics of the donor strain and the match of such characteristics between recipient and donor.

The mean of the lag phase was longer when the recipient received an invader capsule (41 min, SE 8.29) than a colonizer capsule (17 min, SE 5.95), independent of the colonizer/invader status of the recipient. Therefore, strains profited more in terms of growth from a colonizer capsule than from an invader capsule (Fig. 24).

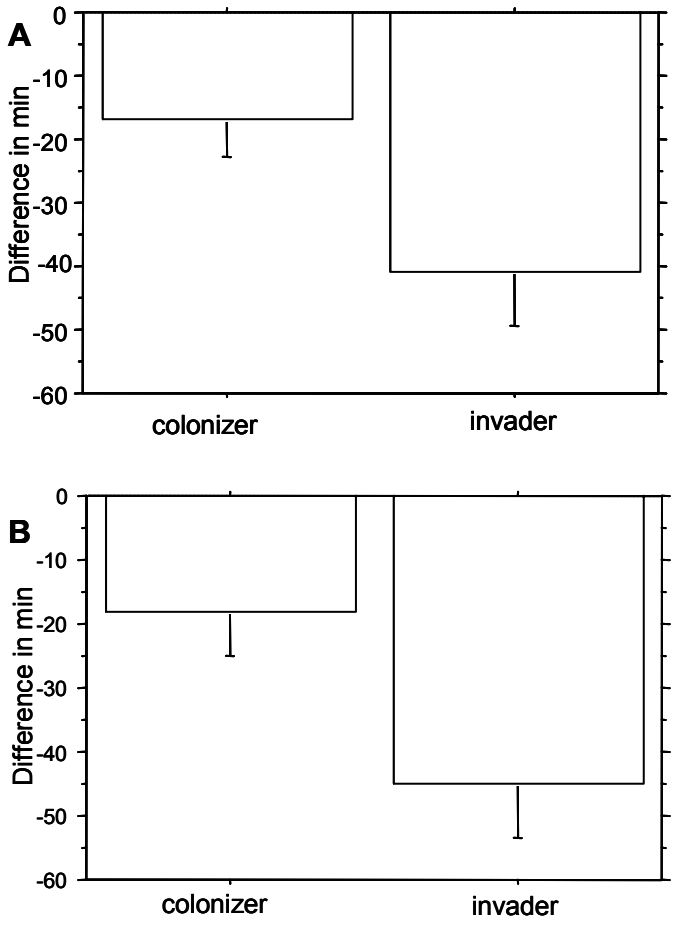


Fig. 24. Differences between the lag phase of the wild-type recipient strains and their capsule mutants stratified according to the invader or colonizer status of the capsule donor.
A) All 110 capsule switch mutants: The difference between colonizer (17 min, SE ± 5.95) and invader (41 min, SE ± 8.29) donors was statistically significant, p-value 0.0164.
B) Excluding 14 capsule switch mutants, for which the recipient and the donor strain showed the same RFLP type, i.e. they were clonally related. The difference between colonizer (18 min, SE ± 6.60) and invader (44 min, SE ± 8.69) donors was statistically significant, p-value 0.0147.
SE: standard error

When a colonizer recipient obtained a new capsule the change of the lag phase was less prolonged (20 min, SE 5.74) than if an invader recipient received a new capsule (29 min, SE 6.69) (although this difference did not reach statistical significance) (Fig. 25).

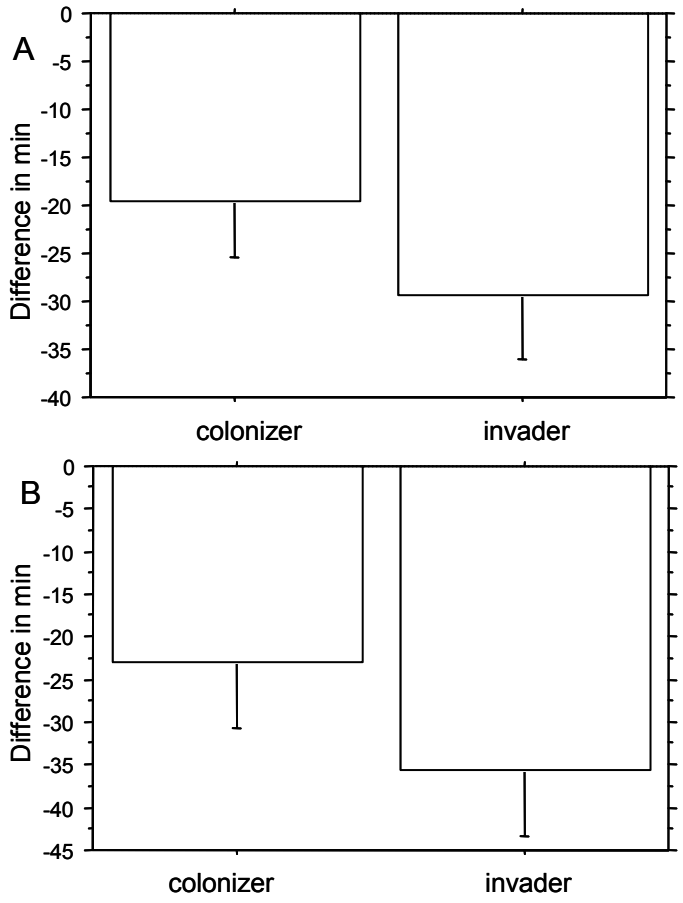


Fig. 25. Differences between the lag phase of the wild-type recipient strains and their capsule mutants stratified according to the invader or colonizer status of the capsule recipients.
A) All 110 capsule switch mutants. The difference between colonizer (20 min, SE ± 5.74) and invader (29 min, SE ± 6.69) recipient was statistically not significant, p-value 0.273.
B) Excluding 14 capsule switch mutants, for which the recipient and the donor srain showed the same RFLP type, i.e. they were clonally related. The difference between colonizer (23 min, SE ± 7.59) and invader (36 min, SE ± 7.72) recipient was statistically not significant, p-value 0.244.
SE: standard error

Transformation of a colonizer capsule into a colonizer recipient caused the smallest extra delay in the lag phase (10 min, SE 7.84). Transformation of an invader capsule into a colonizer recipient, however, was a relative disadvantage (extra delay 37 min, SE 10.98) compared to receiving a colonizer capsule. Transformation of an invader capsule into an invader recipient caused the largest extra delay (46 min, SE 12.85).

Transformation of a colonizer capsule into an invader recipient was a relative advantage (extra delay 23 min, SE 8.83) as compared to receiving an invader capsule (Fig. 26).

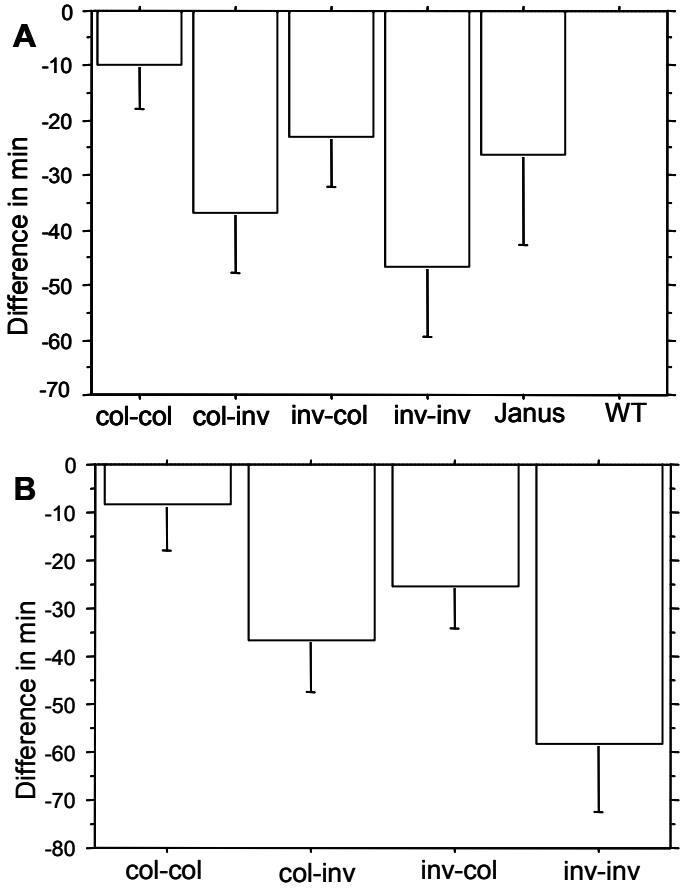


Fig. 26. Differences between the lag phase of the wild-type recipient strains and the capsule switch mutants (extra delay) restricted to recipient-donor pairs.

A) All 110 capsule switch mutants: col-col 10 min, SE 7.84; col-inv 37 min, SE 10.98; inv-col 23 min, SE 8.83; inv-inv 47 min, SE 12.85. Janus: recipients with Janus cassette i.e. without capsule 26 min SE 16.47; WT: wildtype recipient.
P-values compare the following groups: col-col, col-inv 0.472; col-col, inv-col 0.287; col-col, inv-inv 0.011; col-col, Janus 0.340; col-col, WT 0.557, col-inv, inv-col 0.301; col-inv, inv-inv 0.510; col-inv, Janus 0.544; col-inv, WT 0.037; inv-col, inv-inv 0.097; inv-col, Janus 0.859; inv-col, WT 0.168; inv-inv, Janus 0.262; inv-inv, WT 0.012; Janus, WT 0.200.

B) Excluding 14 capsule switch mutants, for which the recipient and the donor srain showed the same RFLP type, i.e. they were clonally related: col-col 8 min, SE 9.73; col-inv 37 min, SE 10.98; inv-col 25 min, SE 8.82; inv-inv 59 min, SE 13.98.
P-values compare the following groups: col-col, col-inv 0.058; col-col, inv-col 0.222; col-col, inv-inv 0.004; col-inv, inv-col 0.406; col-inv, inv-inv 0.197; inv-col, inv-inv 0.041.
Col-col: colonizer recipients with colonizer capsules, inv-col: invader recipients with colonizer capsules, col-inv: colonizer recipients with invader capsules, inv-inv: invader recipients with invader capsules. SE: standard error.

4.3. Discussion

4.3.1 Influence of capsule gene *ugd* on growth and sugar metabolism

Failing of the construction of the single gene *ugd* knockout mutant

The aim was to construct a single gene *ugd* mutant and its complemented strain. With these mutants experiments as growth curves with different carbon sources and measuring cell wall integritiy were planned. Unfortunately the progress of constructing the single gene *ugd* knockout mutant was only partially not successful until now. After transformation of D39SmR with *cpsJ*-Janus-*cpsP*, this DNA construct was not on the right place and the *ugd* gene was already in the capsule locus.

A recent publication explains why the construction of the mutant D39SmRΔ*ugd*::Janus may have failed [112]. Xayarath et al. described in their experiment that deletion of *cps2K* is detrimental to the cell, and such mutants can be isolated only in the presence of suppressor mutations that reduce or eliminate capsule synthesis. Occurrence of *cps2K* deletion mutants seems to be a rare event ($\leq 1/10^6$). This result was consistent with the necessity to transform the rare spontaneous mutants that contained *cps2E* or other suppressor mutations that allowed for survivial in the presence of a *csp2K* deletion [112]. We assume that these spontaneous suppressor mutations did not occur in our *ugd* knockout mutants and therefore these *ugd* mutants were not able to survive.

Strategies to circumvent the problems

Further experiments will be done for constructing the mutants D39SmRΔ*ugd*::Janus, D39SmRΔ*ugd*::*cpsJ-cpsP* and D39SmRΔ*ugd*::*cpsJ-cpsP*Δ*SPD0423-SPD0429*::*aad9-ugd.*

D39SmRΔ*ugd*::Janus: Insertion of *cpsJ*-Janus-*cpsP* in D39SmR with increased growth time during transformation to gain more transformants to allow for more suppressor mutants. I have doubts whether this procedure is successful, because I have already prolonged the growth time for transformants. Additionally, the transformants will be plated out in higher cell concentrations on kanamycin plates.

D39SmRΔ*ugd::cpsJ-cpsP*: Insertion of *cpsJ-cpsP* into D39 and select for small, unencapsulated colonies, which were observed by Xayarath et al. [112] as a property of *cps2K* mutants.

Another theoretical possibility to receive a strain without the function of *ugd* in the capsule locus will be to block the respective gene function by siRNA [146]. However this procedure will be very time consuming and no experience exists in working of gene blocking by siRNA.

D39SmRΔ*ugd*::*cpsJ-cpsP*Δ*SPD0423-SPD0429*::*aad9-ugd*: Knocking out the *ugd* gene of the capsule locus by insertion of *cpsJ*-Janus-*cpsP* DNA fragment into the *ugd* complemented strain D39Δ*SPD0423-SPD0429*::*aad9-ugd.* In D39Δ*SPD0423-SPD0429*::*aad9-ugd* the *ugd* gene was inserted into the *lacE* operon and therefore suppressor mutations by lacking of *ugd* as described by Xayarath et al [112] should not appear. Because of this *ugd* substitution in *lacE* a higher transformation rate will be expected. In a second step the *cpsJ*-Janus-*cpsP* construct will be replaced with *cpsJ-cpsP* to receive a single gene *ugd* in frame mutant.

4.3.2 Growth curves in different culture media

One aim of my project was to test for the role of different carbon sources in capsule gene deficient mutants. Under laboratory conditions pneumococcus is usually grown in rich media such as BHI. However, such media are not optimal for growth experiments since they are undefined and their composition can change substantially between different lots. Therefore, we searched the pneumococcal literature for the choice of different culture media with the aim to identify chemically defined media. In such media the influence of supplementation with different sugars or aminoacids should be better interpretable than in a rich and undefined medium. Two chemically defined media were found, Cden (Annex, table A3) [193] and CDM medium (Annex, table A4) [194].

Unfortunately, six clinical isolates and six capsule switch mutants did not show growth (did not reach OD_{600nm} 0.2 in 450 min) in Cden and CDM. Therefore these media were not used for further experiments.

Instead, growth was evaluated in the semisynthetic medium Lacks (Annex, table A6, A8) and compared to growth in the nutritious and undefined media BHI with an emphasis on the length of the lag phase (defined as the time to reach OD_{600n} 0.2) since this parameter was of interest for our hypothesis.

Growth in Lacks with different supplements showed some common and some individual behaviour among the five tested strains. Addition of sugar was essential for

growth (except maybe for the unencapsulated laboratory strain R6) and glucose and sucrose tended to shorten the lag phase more than maltose and lactose. This is not an unexpected finding. It is known that *S. pneumoniae* depends on external sugars for its energy requirement [124]. Glucose and sucrose are also called “repressing sugars” [126], since the presence of either glucose or sucrose represses lactose-inducible β-galactosidase. Only when “inducer sugars” (lactose, maltose, raffinose or cellobiose) are present as a sole carbon source, carbon catabolite protein A represses CCR. It is possible that the longer lag phase observed in our experiments in association with maltose or lactose as the only carbon source indicates repression of CCR. The laboratory strain D39 and the clinical non-encapsulated strain 110.58 showed some peculiarities. In D39 the addition of sugars enhanced growth only in combination with FCS, but the presence of BSA did not influence growth. In contrast, in the clinical non-encapsulated strain 110.58 removal of BSA enhanced growth as long as sugar(s) were present and supplementation with FCS had no measurable effect. Early, Rane et al. [212] already described the individuality of pneumococcal strains for aminoacids requirements. In their experience aminoacids necessary for the growth of one strain sometimes proved to be inhibitory or indifferent for another strain. It may be that supplementation with BSA in strain 110.58 leads to an over-saturation of aminoacids as described by Rane et al. [212]. It is also possible, that the distinct behaviour of strain D39 reflects adaptive phenomena that happened of decades of growth under laboratory conditions and in rich media. But, it remains to be explained why strain R6, a spontaneous laboratory mutant of D39 does not show the same behaviour. It may be that the lack expressing a polysaccharide capsule lowers the energy requirements of this strain.

All strains did grow in BHI. But, supplementation with FCS helped growth in all tested strains. It is currently, completely unknown, which component or combination of components in FCS mediates this effect. The lack of an effect after addition of sugar or even an inhibitory effect after addition of MEM may be explained by the already rich composition of BHI. The concentrations dependent inhibitory may be explained by changes in pH and osmolarity. *S. pneumoniae* is a fastidious bacterium and changes in pH (optimal pH 7.8 with range of 6.5 - 8.3) can limit the growth [196, 213]. However in stationary phase, when acid products of the fermentation of glucose

arise, the addition of $NaHCO_3$ may be useful as buffer to stable pH value in the medium.
Supplementation of Lacks or BHI with NAD showed only a small effect. The pyridine nucleotide NAD plays a vital role as signal transducer in metabolic conversion and in cellular defence systems. This coenzyme participates as electron carrier in energy transduction and biosynthetic processes. Its oxidized form, NAD^+ has also been identified as important element of regulatory pathways [214]. We cannot exclude that in our experiments the amount of NAD added was not high enough to mediate a measurable effect on growth.

In conclusion, growth experiments showed that chemically defined media do not support growth of diverse clinical strains and are therefore not suitable for our project. The semi-defined medium Lacks may be very useful to test the influence of carbon sources. A limitation in such experiments may be that individual clinical strains may differ for their requirement or tolerance of protein supplement in this medium.
We were not able so far to identify the component contained in FCS that helps growth of pneumococcal strains in BHI, a very consistent phenomenon.

4.3.3 Transformability

Transformational recombination is the main mechanism by which *S. pneumoniae* obtains genetic variation that permits adaptation to changes in the environment. Natural transformation of the capsule locus leading to changes in polysaccharide structure and immunogenicity was observed in *S. pneumoniae* [22]. Uncovering the mechanisms that drive capsule switching is important for a better understanding of the interactions between the host and the pathogen and among different strains of pathogens competing for the host [17].
Our experiments showed that transformation efficacy was higher when capsules were exchanged between two colonizer strains or between two invader strains as compared to the transformation rate between a colonizer and an invader or vice versa. One explanation for this observation may be that genetic homology is higher among colonizer and among invader strains than between colonizer and invader

strains, although pneumococcal isolates show generally high homology for the capsule flanking regions [1, 108].

Another, possible reason might be that activity of the RecA protein, which catalyzes homologous recombination between the internalized DNA and the recipient genome [215] may show some strain specificity. It has been shown that competence may be a response of *S. pneumoniae* to environmental stress [216]. Our transformation experiments were performed in vitro always under the same conditions without stress factors. Therefore we exclude environmental influences like stress factors during transformations as explanation for our results.

Along our hypothesis, the ability of pneumococci to transform from one serotype to another might depend on the presence of genetic information in the chromosomal background which is needed for the expression of the new serotype. Since colonizers are more adapted to survive in the nasopharyngeal niche, their genetic chromosomal background may support the expression of different colonizer capsules better than the capsule of an invader strain.

Capsule operons of colonizers strains were on the average of lower size (19626 bp) than the average size of invader strains (21331bp). A possible explanation may be that for invaders the expression of a capsule is important for survival in the blood [26]. For colonizers the expression of a thick capsule seems to be disadvantageous, because of its inhibitory effect in adherence [120].

We analysed, whether the size of the capsule operon may have influenced the transformation rate. Indeed, the results showed that colonizers accepted more often capsules of bigger sizes than their own capsule, especially when the capsule came from a colonizer (72.4 %). Invaders tended to prefer capsules that were smaller than their one, especially when they came from an invader (70.0%, although this comparison did not reach statistical significance). A reason could be that colonizers are more adapted to the nasopharyngeal space and have a greater ability to respond to environmental changes, which is reflected by their preference for up-take of larger amounts of DNA.

4.3.4 Effect of capsule switch mutants on growth

Our main hypothesis was that serotype specific capsule characteristics such as the colonizer and invader phenotype can be transformed by exchange of the capsule.

We used growth, i.e. the length of the lag phase (time to reach OD_{600nm} 0.2) as a surrogate. Experiments with 110 capsule switch mutants showed, that in general the transformation of capsule genes tended to prolong the lag phase in the mutant strains as compared to the wild type. The reasons for this fitness costs are unclear. Fitness costs have described after the acquisition of mutated *pbp* genes in *S. pneumoniae* mediating β-lactam resistance [61]. It is believed that such fitness cost relate to a disturbance of the fine tuned cell wall machinery and have to be compensated by additional adaptive mutations. Capsule transformation may have similar effects if our hypothesis is true that capsule genes have a role for the central metabolism.

An intriguing finding was the influence of the recipient-donor combination on the change of the lag phase in the capsule switch mutant. The change of the lag phase was greater when the recipient received an invader capsule as compared to a colonizer capsule. These results are consistent with our hypothesis that colonizer capsules help growth [102]. In addition, colonizer recipients which received a colonizer capsule showed the shortest prolongation of the lag phase, while the change was highest when invader recipients received an invader capsule. This observation supports our hypothesis that the capsule plays a role for central metabolism and influence the ability of a strain to colonize the nasopharynx.

In our experiments the length of the lag phase did not correlated with the size of the new capsule. Trzcinski et al. described a significant increase in growth rate for two transformants which received a smaller capsule than their recipient [188]. They speculated that the smaller size of the capsule operon of the donor might reflect lesser complexity and reduced energetic cost of capsular polysaccharide biosynthesis in the capsule switch mutants.

Our results show variability in growth among colonizer or invader and also within the same serotype. It is well known, that pneumococci show genetic diversity and undergo intra- and interspecies genetic exchange [217 - 219], which may be a reason for this variability. Recently, genetic differences were confirmed between strains of the same serotype and with the same multilocus sequence type [220]. Additional diversity may come from cell-to-cell heterogeneity at gene expression and growth rate levels [186, 221 - 223]. Therefore, some variability in growth by strain was not surprising.

4.4. Outlook

Future research with capsule switch mutants

Our results showed that colonization characteristics concerning growth can be at least partly transferred by capsule exchange in *S. pneumoniae*. It would be of great interest to test the capsule switch mutants in adherence and colonization in an animal model. We would expect a higher colonization capacity of recipients, which obtain a colonizer capsule. To compare colonizer recipients with invader or colonizer donors should give an insight in contribution of the genetic background in colonization.

To investigate differences of a colonizer or invader capsule on the sugar metabolism, growth curves could be performed in the semi defined Lacks medium, supplemented with different sugars, by offering the capsule switch mutants and their original clinical isolates different carbon sources. Perhaps the addition of different sugars may shorten the differences in the lag phase observed in the capsule switch mutants and therefore the supplemented sugars may compensate the fitness deficit, which was due to the new capsule.

By measuring the gene expression with microarray assays in the capsule switch mutants and their clinical isolates, more information could be gained about the origin of enzymes and monosaccharides, which are involved in the biosynthesis of the capsule operon.

Further experiments by measuring spontaneous and antibiotic induced lysis to compare the cell wall integrity of capsule switch mutants and their original clinical isolates may give insights whether cell wall synthesis is influenced by another capsule.

5. References

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7. Curriculum vitae

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Schools/apprenticeship

1973 – 1977	Primary School in Münchenbuchsee
1978 – 1983	Secondary School in Hindelbank
1984	traineeship in Hôptial de Lavaux, Cully VD
1985 – 1987	apprenticeship medical assistant in Bern
1987 – 1990	worked as a medical assistant in Ostermundigen, Dr. med. F. Schmid
1991 – 1999	Zollikofen, Dres. med. S. + V. Verones
1989 – 1991	apprenticeship as vocational school teacher
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Languages

German	Native language
French	Good knowledge of written and spoken French
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Academic degrees

1997 – 2000	Graduated from AKAD and Gymnasium Neufeld Bern Matura, Type D
2001	First pre-diploma in scientific subjects, University of Bern
2002	Second pre-diploma in pharmaceutical subjects, University of Bern
2003	Graduated as assistant in pharmacy, University of Bern
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Education/Research

2000 – 2005	Studies of pharmacy at the University of Bern and Basel
2003	worked in the pharmacy of Dr. Beat Wittwer in Laupen and in the Institute for hospital pharmacy, Inselspital Bern as a student of pharmacy and learnt the preparation of drugs and analysis of chemicals and drugs.
2004	Practical training as a student of pharmacy in the hospital pharmacy, Lindenhofspital Bern (10 weeks).
2005 (February – June)	Trained in clinical microbiology as a diploma student in the Laboratory of Prof. Dr. K. Mühlemann at the Institute of Infectious Diseases Bern in collaboration with the University of Basel: *Antibiotic tolerance in clinical isolates of S. pneumoniae*
January 2006 - present	Interfaculty PhD-doctorate (Graduate School for Cellular and Biomedical Sciences) of the University of Bern at the Institute for Infectious Diseases under the supervision of Prof. Dr. K. Mühlemann: *Streptococcus pneumoniae:* *Heteroresistance to penicillin* *Roles of the polysaccharide capsule genes in growth and colonization*

Courses, lectures and exams (PhD)

2006 - 2007	Lectures in clinical microbiology
2006 – 2008	Lectures in clinical infectious diseases
2006 – 2008	Research seminars
2006	Lectures in molecular cell biology
2007	Practical course in Molecular Biological Methods in clinical Diagnosis, Tiefenau Laboratory, Bern
2007	Molecular Cell Microbiology, exam oral Supervisor: Prof. A. Ziemiecki, Prof. A. C. Andres, Tiefenau laboratory, Bern
2007	Clinical Microbiology, exam oral Supervisor: Dr. phil. nat. F. Suter, Dr. pharm. S. Bigler Institute for Infectious Diseases
2008	Colloquium on selected publications, exam oral Supervisor: Prof. J. Frey, Prof. K. Mühlemann Institute for Infectiouse Diseases

Publications

August 2007	Morand B., K. Mühlemann. Heteroresistance to penicillin in *S. pneumoniae.* Proc Natl Acad Sci USA 2007 Aug 28:104(35): 14098-14103.

Presentations at meetings

2007	Roles of *S. pneumoniae* polysaccharide capsule genes in growth and colonization. Oral presentation, 1st Graduate School Students Symposium
2008	Heteroresistance to penicillin in *S. pneumoniae.* Poster presentation, 2st. Graduate School Students Symposium

Attendance at meetings

June 2006	ASM Conference on Streptococcal Genetics, St. Malo France
April 2007	Europneumo 2007 – 8th European Meeting on the Molecular Biology of the Pneumococcus, Oeiras; Portugal
June 2008	6th International Symposium on Pneumococci and Pneumococcal Diseases, Reykjavik, Iceland

Membership of societies

American Society for Microbiology

8. Annex

Table A1. Primers used for construction and control of *ugd* knockout and complemented mutant

Primer	Sequence	Description
cpsJ-BM47_F1	gtagttatttgcattttgtggtgtagc	Amplification of *cpsJ* and *cpsJ-cpsP* fusion product
cpsJ_B_B1_BamHI	cgcggatcctaggataaagaatataactattttccaaccat	Amplification of *cpsJ*
cpsP_C_F1ApaI	tttgggcccacgtgatatttttgaaagagattaatttagtat	Amplification of *cpsP*
cpsP_BM54	gactgtcttaggctgcagaccatact	Amplification of *cpsP* and *cpsJ-cpsP* fusion product
DAM406_BamHI	tctatgcctattccagaggaaatggat	Amplification of Janus cassette
DAM351_ApaI	ctagggcccctttccttatgcttttggac	Amplification of Janus cassette
DAM406_cj_f	tacgagtattgaaaggagaaaatctatgcctattccagaggaaatggat	Amplification of Janus cassette with overlap primer
DAM351_ov_cp_b	cttctaacaattatttttttcatctagggccccttccttatgctttttgg	Amplification of Janus cassette with overlap primer
cpsJ_F12	ttttttccgcggttaaagattca	Amplification of *cpsJ* for transformation, control of correct size of *cpsJ*-Janus_*cpsP* (PCR1*), control of *ugd* in D39 (PCR 4*)
cpsJBM113DAM406b	catttttctcctttcaatactcgta	Amplification of *cpsJ* with overlap primer for fusion with Janus
cpsPBM114_F1	atgaaaaaaataattgttagaagcggtc	Amplification of cpsP with overlap primer for fusion with Janus
cpsP_B1	tctgtgaagcttcttcgtggtattt	Amplification of *cpsP* with overlap primer, Control of correct place downstream of *cpsP* (PCR3*)
cpsP_BM54_b1	gactgtcttaggctgcagaccatact	Control of correct size of *cpsJ*-Janus-*cpsP* (PCR13*)
cpsJ_F1	ttatttcaattaatatttctgaaggggtt	Control of correct place upstream of *cpsJ* (PCR 2*)
kan-mitte_B1	ttcggctaagcggctgtctaagctattcgtata	Control of correct place upstream of *cpsJ* (PCR 2*)
rpsL-mitte_F1	aaaaaacctaactcagcccttcgtaa	Control of correct place downstream of *cpsP* (PCR 3*)
ugd_start_B1	caatcacaacaaaatccgcatct	Control of *ugd* in D39 (PCR 4*), Control of *aad9-ugd* construct (PCR III*)
cps2P_B2	ccggaattctaaacgtccacagaagataacctt	Control of correct place downstream of *cpsP* (PCR 3*)
cps2L_B1	ccggaattcccaatcaaacggagcagatac	Control of correct place downstream of *cpsP* (PCR 3*)
kan_end_F1	aatattatattttactggatgaattgttttag	Control of correct place downstream of *cpsP* (PCR 3*)
cpsJ_BM113_b1	catttttctcctttcaatactcgta	Amplification of *cpsJ* with overlap primer for fusion with *cpsP*
BM113part_BM114F1	tacgagtattgaaaggagaaaaatgaaaaaaataattgttagaagcggtc	Amplificationof *cpsP* with overlap primer for fusion with *cpsP*

*Number belongs to the PCR's in figures 5 to 11.

Table A1. continued: Primers used for construction and control of *ugd* knockout and complemented mutant

Primer	Sequence	Description
cpsJ_end_F1	caagttgtgttattcttattattagttatgttatttta	sequencing of *cpsJ-cpsP*
cpsP_start_B1	gctgagcgttcccattgc	sequencing of *cpsJ-cpsP*
endaad9_ugd_F1	cagattaaaaaaattataaaaaaattgagatctgggta cgagtattgaaaggagaaaaatg	Amplification of ugd with overlap primer for fusion with *aad9*
ugd_B2	ttatttttttcatttttattatactaaattaatctctt	Amplification of *ugd* gene and *aad9-ugd* fusion product
spcF1	cccagatctaccgctctagaactagtggatccc	Amplification of *aad9* and *aad9-ugd* fusion product
spcR1	cccagatctcaatttttttataatttttttaatctg	Amplification of *aad9*
lacEF1	tttagaggctcctatttttttt	Amplification of *lacE* upstream, Amplification of *lacE*up-*aad9-ugd*-lacdown
lacER1	caccggaactccttttttt	Amplification of *lacE* upstream
lacEsp_F1	aaaaaaaggagttccggtgcccagatctaccgctctag aactagtggatccc	Amplification of *aad9-ugd* product with overlap primer for fusion with *lacE* upstream region
ugd.lacE_F	aagagattaatttagtataataaaaatgaaaaaaataa gctgtgtagtaagtttttcca	Amplification of *lacE* downstream with overlap primer for fusion with *lacE* up-*aad9-ugd*
lacER2	cctatctggtcagtatcgga	Amplification of *lacE* downstream with overlap primer for fusion with *lacE*up-*aad9-ugd* and Amplification of *lacE*up-*aad9-ugd-lacE*down
ugd-mitte_F1	acaacaatccttccttcggctatg	Control of correct place of the *lacE*up-*aad9-ugd-lac*down construct downstream (PCR I*)
lacdown_B2	ctagaggtgatgaaggctgaggtt	Control of correct place of the *lacEup-aad9-ugd-lacdown* construct downstream (PCR I*, IV*)
lacup_F2	aaattataagccgttaaaagggtgtcta	Control of correct place of the *lacEup-aad9-ugd-lacdown* construct upstream (PCR II*, IV*)
aad9start_B1	tgattccacggtaccatttcttg	Control of correct place of the *lacEup-aad9-ugd-lacdown* construct upstream (PCR II*)
aad9endF2	gtatgattttaactatggacacgggtaa	Control of *aad9-ugd* construct (PCR III*)

*Number belongs to the PCR's in figures 5 to 11.

Table A2. Primers used for characterization and control of capsule switch mutants

Primer	Sequence	Description
dexB vorstart	ggatagtaatgaagatggagttggtga	Control of correct place of Janus cassette, LRPCR for control of correct ligation ot capsule to upstream flanking region (LRPCR I*, V*)
kan_B1	gatattctcattttagccatttattatttc	Control of correct place of Janus cassette (PCR I*)
kan_start_F1	aggaaataataaatggctaaaatgagaat	Control of correct size of Janus cassette (PCR II*, III*)
DAM351	ctttccttatgcttttggac	Control of correct size of Janus cassette (PCR II*). Sequencing primer for *str* allele
rpsL_mitte_B1	gaatttacgaagggctgagttaggttt	Control of intact Janus cassette and correct place of the Janus cassette (PCR III*)
rpsL_mitte_F1	aaaaaacctaactcagcccttcgtaa	Control of correct place of the Janus cassette (PCR IV*)
DAM350	accaaaaataaaaaaacacaggag	Sequencing primer for *str* allele
dexBstart2	tttctcccgtttatgacagccctatgg	Amplification of Janus cassette
aliAend2	aagattggacgccctgtacgagatgt	Amplification of Janus cassette
aliAend1b	ctggttcacttgtacctttatttcct	Control of correct place of Janus cassette and control of correct ligation of capsule to downstream capsule flanking reagion (PCR IV*)
dexB-cpsA_REV	ctatctgctaaaacagcgacactga	LRPCR for control of correct ligation of capsule to upstream capsule flanking region (PCR V*)
cpsO_F1	caaatggccaattaggaacgg	Control of correct ligation of capsule to downstream capsule flanking region (PCR VI*)
1430TRZ	tgtccaatgaagagcaagacttgacagtag	LRPCR detection of capsule operon and RFLP (LRPCR VII*)
1402TRZ	caattgtcacgcccgcaagggcaagt	LRPCR detection of capsule operon and RFLP (LRPCR VII*)
LRPdexB_vorstart1	tggatagtaatgaagatggagttggtgatttgco	LRPCR detection of Janus cassette (LRPCR VIII*)
LRPaliA_end1	catgttttgcgagatcttcttgagccotttttatt	LRPCR detection of Janus cassette (LRPCR VIII*)
TTM07_TRZ	ctactgttcaagtcttgctcttcattggaca	LRPCR detection of capsule upstream flanking region for RFLP (LRPCR IX*)
TTM09_TRZ	ctaaaacaggggaaattctggcaacaacgc	LRPCR detection of capsule upstream flanking region for RFLP (LRPCR IX*)
TTM08_TRZ	acttgcccttgcgggcgtgacattattg	LRPCR detection of capsule downstream flanking region for RFLP (LRPCR X*)
TTM10_TRZ	aatcgcgaaacgtcccagccgtggaaactc	LRPCR detection of capsule downstream flanking region for RFLP (LRPCR X*)

*Number belongs to the PCR's in figures 19 to 23.

Table A3. Composition of Cden culture medium [193]

Component	**Amount**
Autoclaved Cden Base	200 ml
Sterile filtered amino acids (His, Tyr, Arg)	50 ml
Glutamine (1 mg/ml)	10 ml
Vitamins-choline	10 ml
Pyruvate 2%	5 ml
SAC	40 ml
Supplement	13 ml
Buffer phosphate pH 8.0	15 ml
Leucine (10 mg/ml)	10 ml
Phenylalanine (10 mg/ml)	5 ml
Lysine (10 mg/ml)	9 ml
Choline (1 mg/ml) or Ethanolamine (2 mg/ml)	2 ml/8 ml
sterile water	up to 400ml
Cden base (composition of components of Cden)	
Glycine	190 mg
Alanine	350 mg
Valine	720 mg
Isoleucine	760 mg
Proline	1160 mg
Serine	590 mg
Threonine	450 mg
Methionine	310 mg
Triptophan	140 mg
Aspartic Acid	720 mg
Glutamic Acid	2200 mg
Cysteine	150 mg
H2Od	up to 2L
Adjust pH to 7.0, sterilize by autoclaved.	
Amino acids (composition of components of Cden)	
L-Histidine	640 mg
L-Tyrosine	122 mg
L-Arginine (mono-hydro)	800 mg
H_2Od	up to 1L
Filtration	
Vitamins-choline (composition of components of Cden)	
Adams I	12.8 ml
Asparagine (5 mg/ml)	32 ml
H_2Od	36 ml
Adams I (composition of components of Cden)	
Biotin (0.5 mg/ml)	0.06 ml
Nicotinic Acid	30 mg
Pyridoxin-chlorhydrate	35 mg
Calcium Pantothenate	120 mg
Thiamine-HCl	32 mg
Riboflavin	14 mg
H_2Od	up to 200 ml
Filtration	

Table A3. continued: Composition of Cden culture medium [193]

SAC (composition of components of Cden medium)	
Sodium acetate · 3 H_2O	20 g
Sodium chlorate	20 g
H_2Od	up to 1L
Supplement (composition of components of Cden medium)	
Salts mixture I	20 ml
Glucose 20%	40 ml
Saccharose 50%	2 ml
Adenosine (2 mg/ml)	40 ml
Uridine (2 mg/ml)	40 ml
Salts mixture I (composition of components of Cden medium)	
$MgCl_2 \cdot 6H_2O$	100 g
$CaCl_2 \cdot 2H_2O$	0.662 g
$SO_4Mn \cdot H_2O$ 0.1 M	0.2 ml
H_2Od	up to 1 L

Table A4. Composition of CDM culture medium [194, 195]

	Component	Concn (mg/liter)
1.	$FeSO_4 \cdot 7H_20$	5
	$Fe(NO_3)_2 \cdot 9H_2O$	1
	K_2HPO_4	200
	KH_2PO_4	1,000
	$MgSO_4 \cdot 7H_2O$	700
	$MnSO_4$	5
2.	DL-Alanine	100
	L-Arginine	100
	L-Aspartic acid	100
	L-Cystine	50
	L-Glutamic acid	100
	L-Glutamine	200
	Glycine	100
	L-Histidine	100
	L-Isoleucine	100
	L-Leucine	100
	L-Lysine	100
	L-Methionine	100
	L-Phenylalanine	100
	L-Proline	100
	Hydroxy-L-proline	100
	L-Serine	100
	L-Threonine	200
	L-Tryptophan	100
	L-Tyrosine	100
	L-Valine	100
3.	*p*-Aminobenzoic acid	0.2
	Biotin	0.2
	Folic acid	0.8
	Niacinamide	1
	β-Nicotinamide adenine dinucleotide	2.5
	Pantothenate calcium salt	2
	Pyridoxal	1
	Pyridoxamine dihydrochloride	1
	Riboflavin	2
	Thiamine hydrochloride	1
	Vitamin B_{12}	0.1
4.	Glucose	10,000
5.	Adenine	20
	Guanine hydrochloride	20
	Uracil	20
6.	$CaCl_2 \cdot 6H_2O$[a]	10
	$NaC_2H_3O_2 \cdot 3H_2O$	4,500
	L-Cysteine	500
	$NaHCO_3$	2,500
	$NaH_2PO_4 \cdot H_2O$	3,195
	Na_2HPO_4	7,350

[a] BDH Chemicals, Ltd., analytical reagent.

Table A5. Composition of Lacks culture medium (1960) [192, 196]

Component	**Amount/litre**
casein hydrolysat (difco vitaminfree casamino-acids)	5g
tryptophan	6 mg
cystine	35 mg
sodium acetate	2 g
K_2HPO_4	8.5 g
$MgCl_2$ x6 H_2O	0.5 g
$CaCl_2$	2.5 mg
$MnSO_4x4H_2O$	25 µg
$FeSO_4x7H_2O$	0.5 µg
$CuSO_4x5H_2O$	0.5 µg
$ZnSO_4x7H_2O$	0.5 µg
biotin	0.2 µg
nicotine acid	0.2 mg
pyridoxin HCl	0.2 mg
thiamin HCl	0.2 mg
riboflavin	0.1 mg
calcium-Pantothenat	0.6 mg
glucose	2 g
BSA 4 %	12 ml
catalase	3000 units
fresh yeast extract	30 ml

Table A6. Composition of modified Lacks culture medium (1966) [197, 198]

Component	**Amount/litre**
casein hydrolysat (acid hydrolyzed casein)	5g
casein hydrolysat (enzymatic casein hydrolysate (Nutritional Biochemicals)	1g
tryptophan	6 mg
cysteine HCl	40 mg
asparagin	50 mg
glutamine	10 mg
choline HCl	5 mg
adenin	5 mg
sodium acetate	2 g
K_2HPO_4	8.5 g
$MgCl_2$ x6 H_2O	0.5 g
$CaCl_2$	0.6 mg
$MnSO_4x4H_2O$	0.2 mg
$FeSO_4x7H_2O$	0.5 mg
$CuSO_4x5H_2O$	0.5 mg
$ZnSO_4x7H_2O$	0.5 mg
$NaHCO_3$	0.4 g
biotin	0.6 ug
nicotine acid	0.3 mg
pyridoxin HCl	0.3 mg
thiamin HCl	0.3 mg
riboflavin	0.14 mg
calcium-pantothenat	1.2 mg
glucose	2 g
BSA	0.5 g
3000 units catalase	3000 units
fres yeast extract	30 ml

Table A7. Composition of CAT culture medium [199, 200]

Component	**Amount/litre**
NaCl	5 g
yeast extract (Difco)*	1 g
tryptone (Difco)*	5 g
enzymatic casein hydrolysate	10 g
choline HCl	5 mg
after autoclaving	
glucose	2 g
K_2HPO_4	1/60 (0.0167M)

*(Becton Dickinson, le Pont de Claix, France)

Table A8. Different culture media for growth curves

Media	Supplementations
BHI	
BHI	FCS 5 %
BHI	MEM 16%
BHI	MEM 32%
BHI	2% $NaHCO_3$ 2.2%
BHI	2% $NaHCO_3$ 4%
BHI	2% $NaHCO_3$ 6%
BHI	2% $NaHCO_3$ 7.5%
BHI	UDP-GlcUA 0.01mM
BHI	UDP-GlcUA 0.1mM
BHI	UDP-GlcUA 0.1mM
BHI	UDP-Glc 1.4mM
BHI	GlcUA 0.01mM
BHI	GlcUA 0.1mM
BHI	GlcUA 1.4mM
BHI	NAD 0.25mg/ml
Lacks[1)]	
Lacks[1)]	FCS 5 %
Lacks[1)]	Glc 10mM
Lacks[1)]	Glc 10mM + 5% FCS
Lacks[1)]	Suc 10mM
Lacks[1)]	Suc 10mM + 5% FCS
Lacks[1)]	Malt 10mM
Lacks[1)]	Malt 10mM + 5% FCS
Lacks[1)]	Lac 10mM
Lacks[1)]	Lac 10mM + 5% FCS
Lacks[1), 2)]	
Lacks[1), 2)]	FCS 5%
Lacks[1), 2)]	Glc 10mM
Lacks[1), 2)]	Glc 10mM + 5%FCS
Lacks[1), 2)]	Suc 10mM
Lacks[1), 2)]	Suc 10mM + 5%FCS
Lacks[1), 2)]	NAD 0.25mg/ml
Lacks[1), 2)]	Suc 10mM with NAD 0.25mg/ml
THY	
THY	FCS 5%
THY	NAD 0.25mg/ml

[1)] Lacks culture medium (1966) (table A6) without Glucose
[2)] Lacks culture medium (1966) (table A6) without Glucose, without BSA

Abbreviations:

BHI	brain heart infusion broth (Becton Dickinson, le Pont de Claix, France)
FCS	fetal calf serum (Biochrom KG, Berlin, Germany)
THY	Tod Hewitt broth with yeast extract (BD, le Pont de Claix, France)
MEM	minimal essential medium (Gibco, Paisley, United Kingdom)
$NaHCO_3$	sodium hydrogencarbonat (Sigma-Aldrich, Buchs, Switzerland)
UDP-GlcUA	UDP-glucuronic acid (Sigma-Aldrich, Buchs, Switzerland)
GlcUA	glucuronic acid (Sigma-Aldrich, Buchs, Switzerland)
UDP-Glc	UDP-glucose (Sigma-Aldrich, Buchs, Switzerland)
NAD	nicotinamide adenine dinucleotide (Fluka, Buchs, Switzerland)
Suc	sucrose (Sigma-Aldrich, Buchs, Switzerland)
Malt	maltose (Sigma-Aldrich, Buchs, Switzerland)
Lac	lactose (Sigma-Aldrich, Buchs, Switzerland)
BSA	bovine serum albumine (Sigma-Aldrich, Buchs, Switzerland)

Capsule switch mutants

Figure legend for table A9 to A12:
The first number : of the capsule switch mutant describes the recipient original clinical isolate, the second number the donor original clinical isolate, b1 after the capsule switch mutant means one backcrosstransformation, b2: two backcrosstransformations. RFLP: restriction fragment length polymorphism, bp: basepairs.

Table A9. Capsule switch mutants, colonizers as donors and invaders as recipients.

DONOR				RECIPIENT				CAPSULE SWITCH MUTANT
colonizer	serotype	RFLP	capsule size bp	invader	serotype	RFLP	capsule size bp	invader - colonizer
203.24	**6A**	11	17677	B103.66	**14**	1	19918	B103.66cps203.24b1
106.66	**6B**	3	17506	B103.66	**14**	1	19918	B103.66cps106.66
207.31	**15**	1	18626	B103.66	**14**	1	19918	B103.66cps207.31
B112.27	**18C**	14	21819	B103.66	**14**	1	19918	B103.66cpsB112.27
B201.73	**19F**	40	19798	B103.66	**14**	1	19918	B103.66cpsB201.73
B112.27	**18C**	14	21819	B110.04	**7F**	8	24127	B110.04cpsB112.27
B201.73	**19F**	40	19798	B110.04	**7F**	8	24127	B110.04cpsB201.73b1
203.29	**23F**	11	22330	B110.04	**7F**	8	24127	B110.04cps203.29
203.24	**6A**	11	17677	208.41	**7F**	8	24127	208.41cps203.24
106.66	**6B**	3	17506	208.41	**7F**	8	24127	208.41cps106.66
108.34	**19F**	39	19798	208.41	**7F**	8	24127	208.41cps108.34
B201.73	**19F**	40	19798	208.41	**7F**	8	24127	208.41cpsB201.73
103.57	**23F**	11	22330	208.41	**7F**	8	24127	208.41cps103.57
203.29	**23F**	11	22330	208.41	**7F**	8	24127	208.41cps203.29
B201.61	**18C**	16	21819	208.41	**7F**	8	24127	208.41cpsB201.61
307.14	**18C**	14	21819	208.41	**7F**	8	24127	208.41cps307.14
B201.73	**18C**	16	21819	B109.15	**7F**	8	24127	B109.15cpsB201.61
307.14	**18C**	14	21819	B109.15	**7F**	8	24127	B109.15cps307.14
203.24	**6A**	11	17677	B109.15	**7F**	8	24127	B109.15cps203.24
207.31	**15**	1	18626	B109.15	**7F**	8	24127	B109.15cps207.31
108.34	**19F**	39	19798	B109.15	**7F**	8	24127	B109.15cps108.34
B201.73	**19F**	40	19798	B109.15	**7F**	8	24127	B109.15cpsB201.73
103.57	**23F**	11	22330	B109.15	**7F**	8	24127	B109.15cps103.57
203.29	**23F**	11	22330	B109.15	**7F**	8	24127	B109.15cps203.29
106.66	**6B**	3	17506	B109.15	**7F**	8	24127	B109.15cps106.66
203.24	**6A**	11	17677	B101.38	**5**	28	19969	B101.38cps203.24
207.31	**15**	1	18626	B101.38	**5**	28	19969	B101.38cps207.31
307.14	**18C**	14	21819	B101.38	**5**	28	19969	B101.38cps307.14
108.34	**19F**	39	19798	B101.38	**5**	28	19969	B101.38cps108.34
B201.73	**19F**	40	19798	B101.38	**5**	28	19969	B101.38cpsB201.73b2
B201.73	**19F**	40	19798	B101.38	**5**	28	19969	B101.38cpsB201.73b1
B201.73	**19F**	40	19798	B101.38	**5**	28	19969	B101.38cpsB201.73
203.29	**6A**	11	17677	B101.38	**5**	28	19969	B101.38cps203.29
103.57	**23F**	11	22330	B101.38	**5**	28	19969	B101.38cps103.57
B201.61	**18C**	16	21819	B101.38	**5**	28	19969	B101.38cpsB201.61
106.66	**6B**	3	17506	B101.38	**5**	28	19969	B101.38cps106.66

Table A10. Capsule switch mutants, invaders as donors and colonizers as recipients.

DONOR				RECIPIENT				CAPSULE SWITCH MUTANT
invader	serotype	RFLP	capsule size bp	colonizer	serotype	RFLP	capsule size bp	colonizer-invader
B101.77	**14**	8	19918	B201.61	**18C**	16	21819	B201.61cpsB101.77
109.74	**9V**	1	20856	B201.61	**18C**	16	21819	B201.61cps109.74
B101.77	**14**	8	19918	307.14	**18C**	14	21819	307.14cpsB101.77_b1
208.41	**7F**	8	24127	307.14	**18C**	14	21819	307.14cps208.41
B101.77	**14**	8	19918	B201.73	**19F**	40	19798	B201.73cpsB101.77
201.12	**14**	5	19918	B201.73	**19F**	40	19798	B201.73cps201.12
201.38	**9V**	1	20856	B201.73	**19F**	40	19798	B201.73cps201.38
109.74	**9V**	1	20856	B201.73	**19F**	40	19798	B201.73cps109.74
208.41	**7F**	8	24127	B201.73	**19F**	40	19798	B201.73cps208.41
203.39	**4**	17	20936	B201.73	**19F**	40	19798	B201.73cps203.39
201.38	**9V**	1	20856	304.78	**6A**	11	17677	304.78cps201.38
201.12	**14**	5	19918	304.78	**6A**	11	17677	304.78cps201.12
B109.15	**7F**	8	24127	304.78	**6A**	11	17677	304.78cpsB109.15
109.74	**9V**	1	20856	304.78	**6A**	11	17677	304.78cps109.74
B101.77	**14**	8	19918	304.78	**6A**	11	17677	304.78cpsB101.77
B103.66	**14**	1	19918	304.78	**6A**	11	17677	304.78cpsB103.66
109.74	**9V**	1	20856	203.24	**6A**	11	17677	203.24cps109.74
201.38	**9V**	1	20856	203.24	**6A**	11	17677	203.24cps201.38
B101.77	**14**	8	19918	203.24	**6A**	11	17677	203.24cpsB101.77
B103.66	**14**	1	19918	203.24	**6A**	11	17677	203.24cpsB103.66
201.38	**9V**	1	20856	106.66	**6B**	3	17506	106.66cps201.38
B109.15	**7F**	8	24127	106.66	**6B**	3	17506	106.66cpsB109.15
208.41	**7F**	8	24127	106.66	**6B**	3	17506	106.66cps208.41
B101.77	**14**	8	19918	106.66	**6B**	3	17506	106.66cpsB101.77
109.74	**9V**	1	20856	106.66	**6B**	3	17506	106.66cps109.74

Table A11. Capsule switch mutants, colonizers as donors and colonizers as recipients.

DONOR				RECIPIENT				CAPSULE SWITCH MUTANT
colonizer	serotype	RFLP	capsule size bp	colonizer	serotype	RFLP	capsule size bp	colonizer-colonizer
203.29	**23F**	11	22330	103.57	**23F**	11	22330	103.57cps203.29
103.57	**23F**	11	22330	103.57	**23F**	11	22330	103.57cps103.57
203.24	**6A**	11	17677	B201.73	**19F**	40	19798	B201.73cps203.24
304.78	**6A**	11	17677	B201.73	**19F**	40	19798	B201.73cps304.78
106.66	**6B**	3	17506	B201.73	**19F**	40	19798	B201.73cps106.66
103.57	**23F**	11	22330	B201.73	**19F**	40	19798	B201.73cps103.57
203.29	**23F**	11	22330	B201.73	**19F**	40	19798	B201.73cps203.29
B112.27	**18C**	14	21819	B201.73	**19F**	40	19798	B201.73cpsB112.27
B201.61	**18C**	16	21819	B201.73	**19F**	40	19798	B201.73cpsB201.61
307.14	**18C**	14	21819	B201.73	**19F**	40	19798	B201.73cps307.14
207.31	**15**	1	18626	B201.73	**19F**	40	19798	B201.73cps207.31
B201.73	**19F**	40	19798	B201.73	**19F**	40	19798	B201.73cpsB201.73
203.29	**23F**	11	22330	B201.61	**18C**	16	21819	B201.61cps203.29
203.24	**6A**	11	17677	304.78	**6A**	11	17677	304.78cps203.24
103.57	**23F**	11	22330	106.66	**6A**	11	17677	304.78cps103.57
B201.73	**23F**	40	22330	106.66	**6A**	11	17677	304.78cpsB201.73
106.66	**6B**	3	17506	106.66	**6A**	11	17677	304.78cps106.66
108.34	**19F**	39	19798	106.66	**6A**	11	17677	304.78cps108.34
304.78	**6A**	11	17677	106.66	**6A**	11	17677	304.78cps304.78
203.24	**6A**	11	17677	203.24	**6A**	11	17677	203.24csp203.24
103.57	**23F**	11	22330	106.66	**6B**	3	17506	106.66cps103.57
203.29	**23F**	11	22330	106.66	**6B**	3	17506	106.66cps203.29
106.66	**6B**	3	17506	106.66	**6B**	3	17506	106.66cps106.66
108.34	**19F**	39	19798	106.66	**6B**	3	17506	106.66cps108.34
111.46	**19F**	4	19798	106.66	**6B**	3	17506	106.66cps111.46
307.14	**18C**	14	21819	106.66	**6B**	3	17506	106.66cps307.14
207.31	**15**	1	18626	106.66	**6B**	3	17506	106.66cps207.31
201.47	**19F**	7	19798	106.66	**6B**	3	17506	106.66cps201.47
B201.73	**19F**	40	19798	106.66	**6B**	3	17506	106.66cpsB201.73
203.24	**6A**	11	17677	106.66	**6B**	3	17506	106.66cps203.24
B112.27	**18C**	14	21819	106.66	**6B**	3	17506	106.66cpsB112.27

Table A12. Capsule switch mutants, invaders as donors and invaders as recipients.

DONOR				RECIPIENT				CAPSULE SWITCH MUTANT
invader	serotype	RFLP	capsule size bp	invader	serotype	RFLP	capsule size bp	invader-invader
B101.38	**5**	28	19969	B103.66	**14**	1	19918	B103.66cpsB101.38
201.12	**14**	5	19918	B103.66	**14**	1	19918	B103.66cps201.12
B103.66	**14**	1	19918	B103.66	**14**	1	19918	B103.66cpsB103.66
B204.27	**4**	17	20936	208.41	**7F**	8	24157	208.41cpsB204.27
B109.15	**7F**	8	24157	208.41	**7F**	8	24157	208.41cpsB109.15
201.12	**14**	5	19918	208.41	**7F**	8	24157	208.41cps201.12
B103.66	**14**	1	19918	208.41	**7F**	8	24157	208.41cpsB103.66
109.74	**9V**	1	20856	208.41	**7F**	8	24157	208.41cps109.74
201.38	**9V**	1	20856	208.41	**7F**	8	24157	208.41cps201.38
208.41	**7F**	8	24157	208.41	**7F**	8	24157	208.41cps208.41
B109.15	**7F**	8	24157	B109.15	**7F**	8	24157	B109.15cpsB109.15
B106.79	**1**	37	22182	B101.38	**5**	28	19969	B101.38cpsB106.79
203.39	**4**	17	20936	B101.38	**5**	28	19969	B101.38cps203.39
208.41	**7F**	8	24157	B101.38	**5**	28	19969	B101.38cps208.41
201.12	**14**	5	19918	B101.38	**5**	28	19969	B101.38cps201.12
109.74	**9V**	1	20856	B101.38	**5**	28	19969	B101.38cps109.74
201.38	**9V**	1	20856	B101.38	**5**	28	19969	B101.38cps201.38
B101.77	**14**	8	19918	B101.38	**5**	28	19969	B101.38cpsB101.77
B203.76	**5**	38	19969	B101.38	**5**	28	19969	B101.38cpsB203.76
B101.38	**5**	28	19969	B101.38	**5**	28	19969	B101.38cpsB101.38

Table 13. Recipient strains with the Janus cassette

RECIPIENT STRAINS WITH JANUS CASSETTE	**RFLP**
304.78Δ*cps*::Janus	11
203.24Δ*cps*::Janus	11
106.66Δ*cps*::Janus	3
B201.61Δ*cps*::Janus	16
307.14Δ*cps*::Janus	14
B201.73Δ*cps*::Janus	40
103.57Δ*cps*::Janus	11
B101.38Δ*cps*::Janus	28
208.41Δ*cps*::Janus	8
B109.15Δ*cps*::Janus	8
B110.04Δ*cps*::Janus	8
B103.66Δ*cps*::Janus	1

Fig. A1. Length of the lag phase of capsule switch mutants in order to test different media. Blue bars: TSB with yeast extract, brown bars: BHI with FCS, light brown bars: BHI

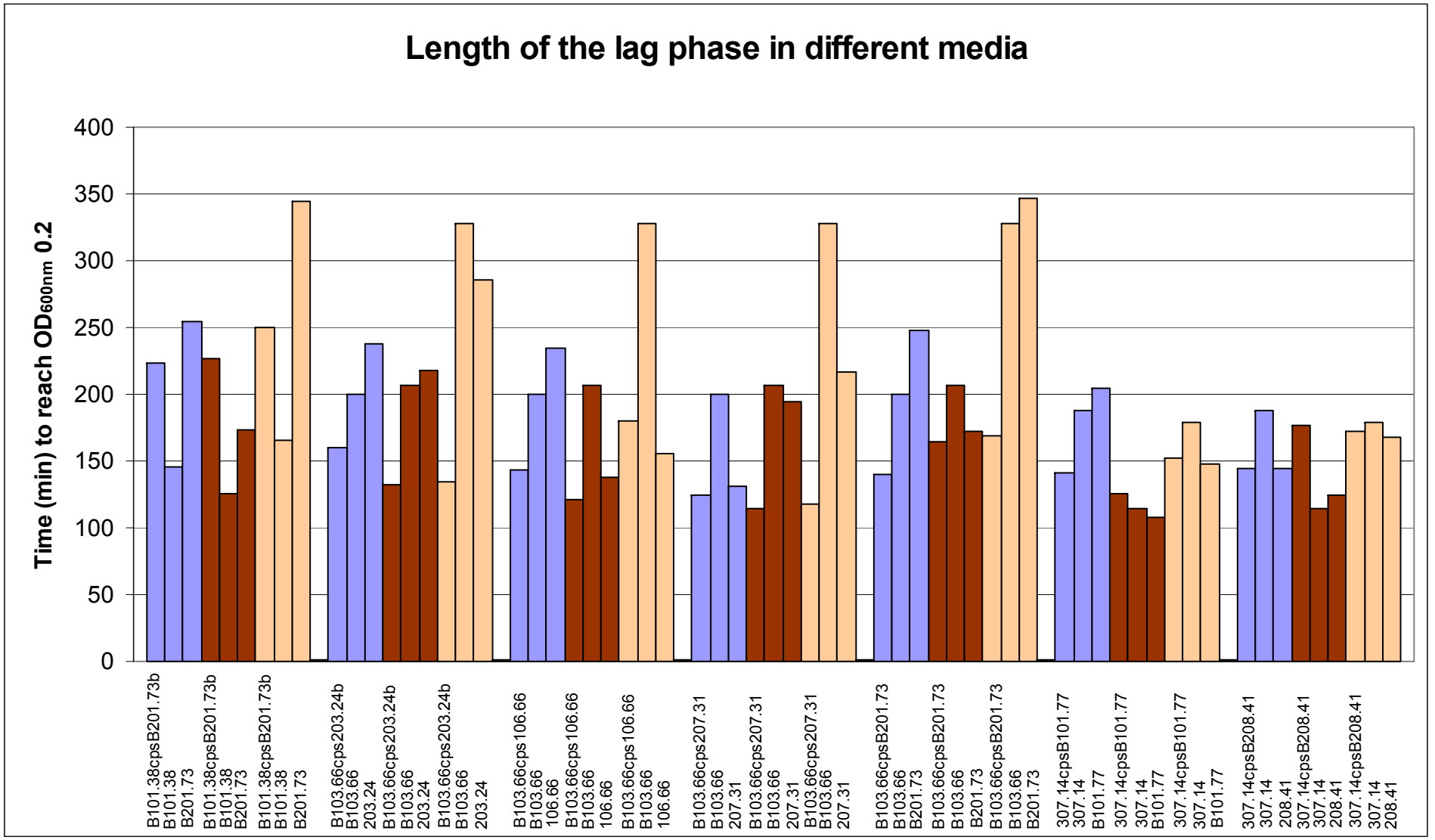
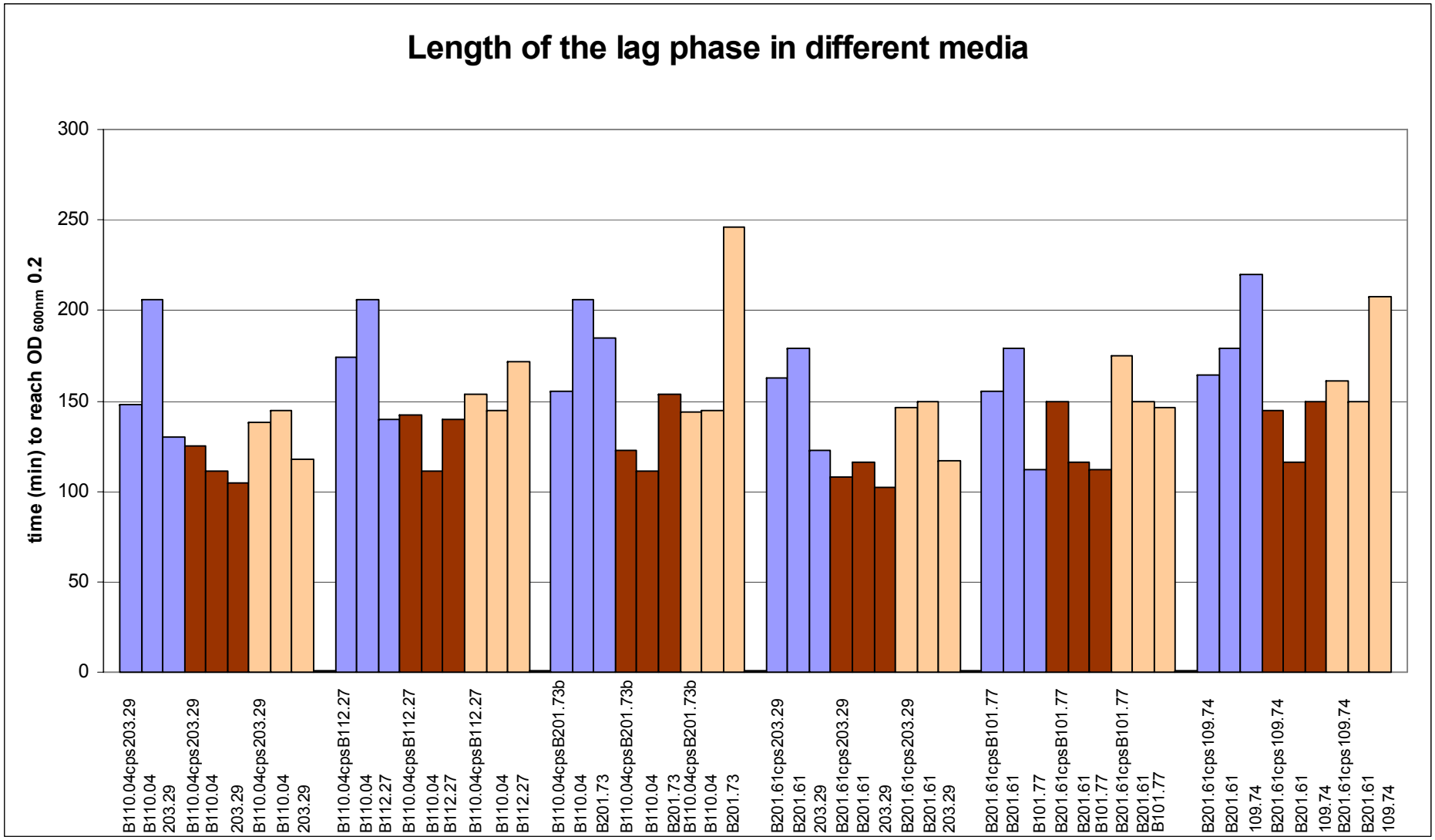


Fig. A2. Length of the lag phase of capsule switch mutants in order to test different media. Blue bars: TSB with yeast extract, brown bars: BHI with FCS, light brown bars: BHI



Length of lag phase of each capsule switch mutant (fig. A3 to A13

Figure legend for figures A3 to A13
Light blue bars: wt colonizer, light yellow bars: wt invader, green bars: wt with Janus cassette, blue bars: colonizer donors, yellow bars: invader donors, hatched blue bars: mean of colonizer donors, hatched yellow bars: mean of invader donors, grey bars: backtransformation i.e. recipient obtains his own capsule.
Description of capsule switch mutants: First number describes the recipient strain, second number the capsule donor strain. Behind the strain descripition the serotype is added of the donor capsule in the capsule switch mutants.
Mean colonizer describes the average of all colonizer capsule donors; mean invader describes the average of all invader capsule donors.
Error bars: standard deviation (SD)

Fig. A3. Length of the lag phase of recipient 304.78 serotype 6A with different capsules

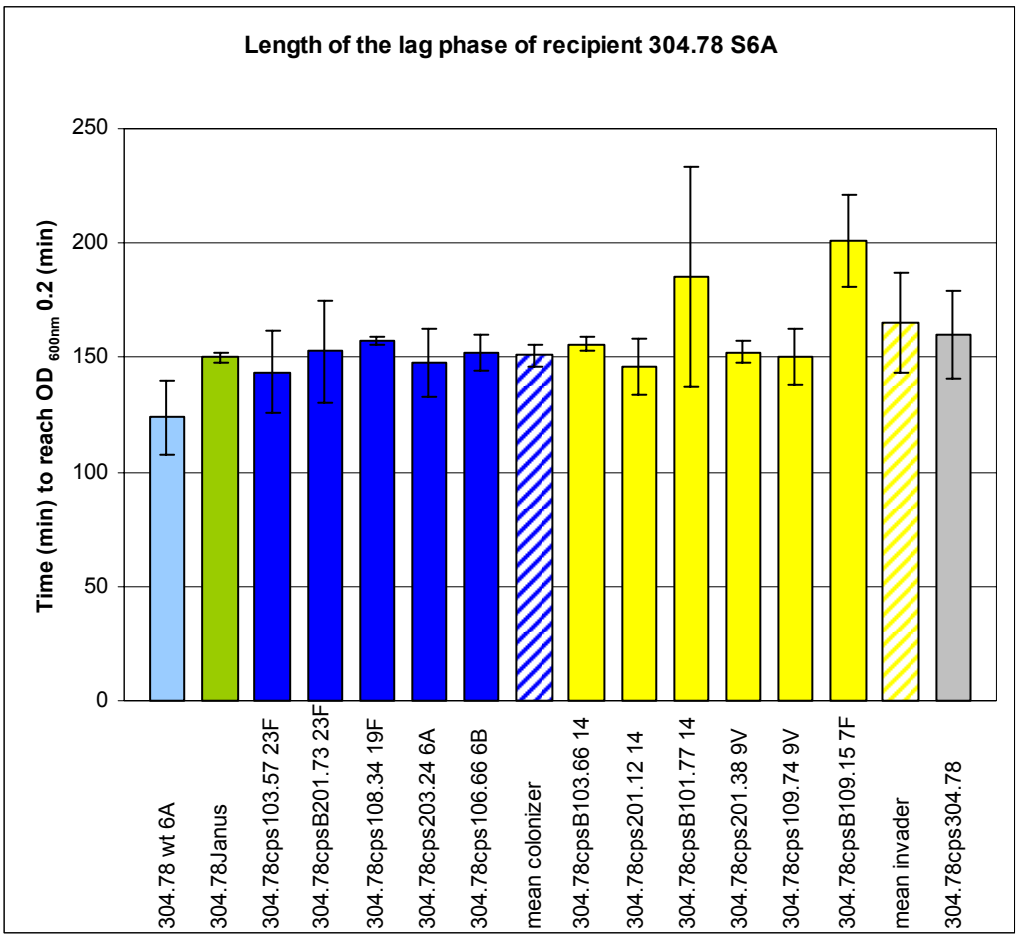


Fig. A4. Length of the lag phase of recipient 203.24 serotype 6A with different capsules

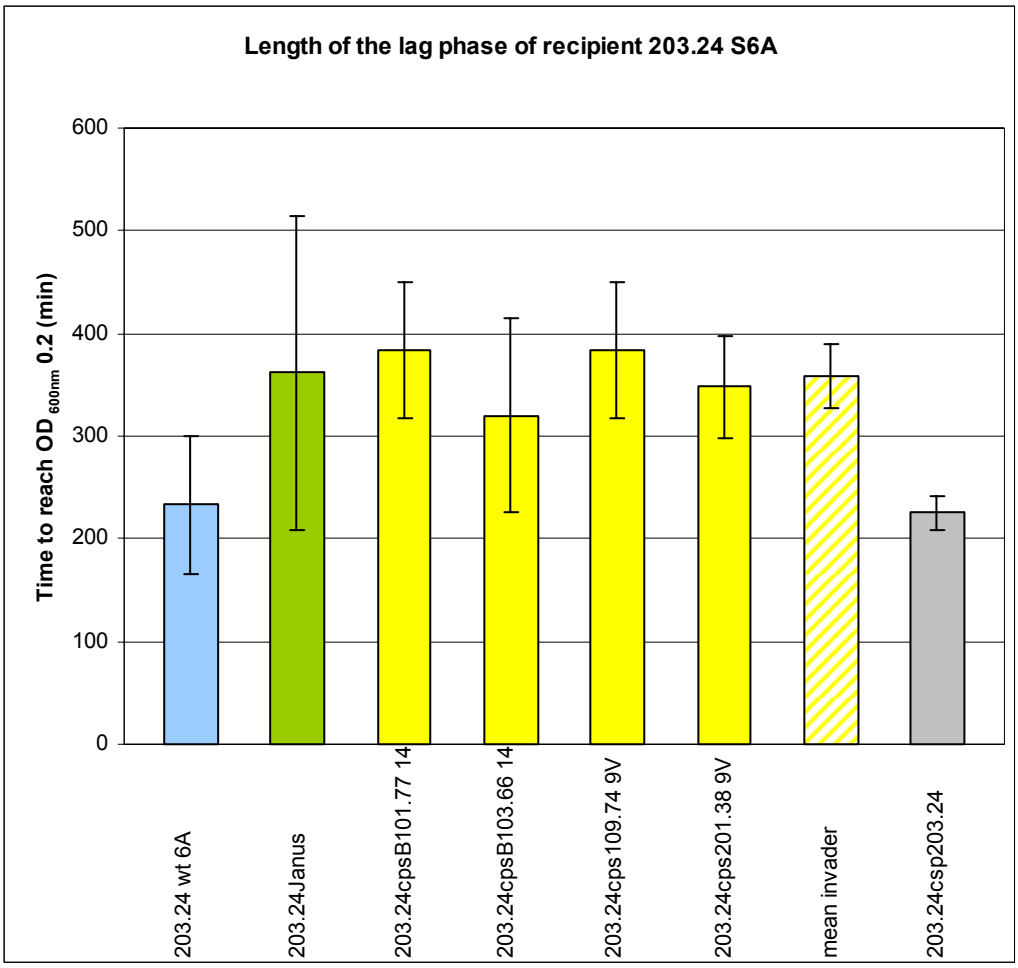


Fig. A5. Length of the lag phase of recipient 106.66 serotype 6B with different capsules

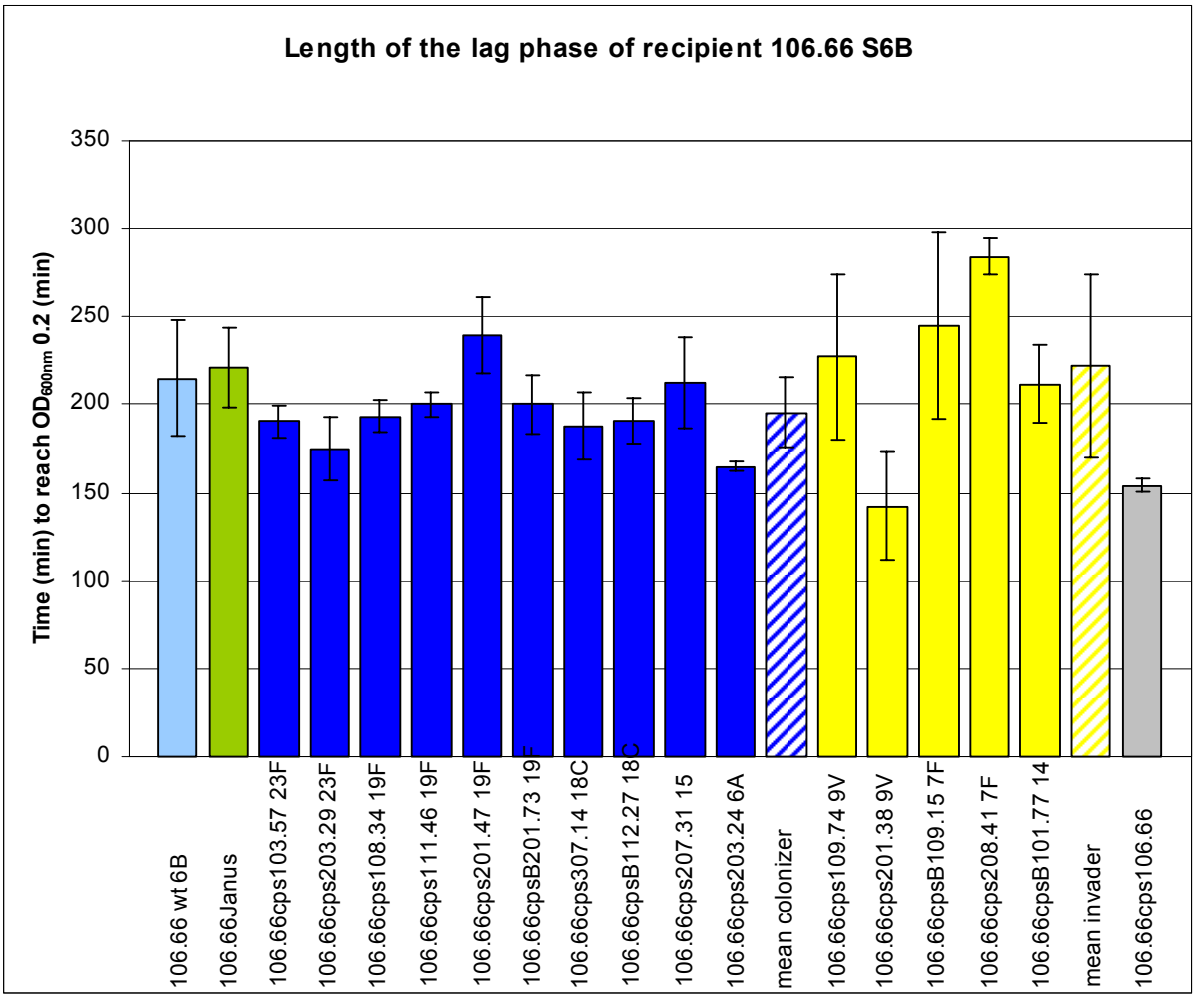


Fig. A6. Length of the lag phase of recipients B201.61 and 307.14, both serotype 18C

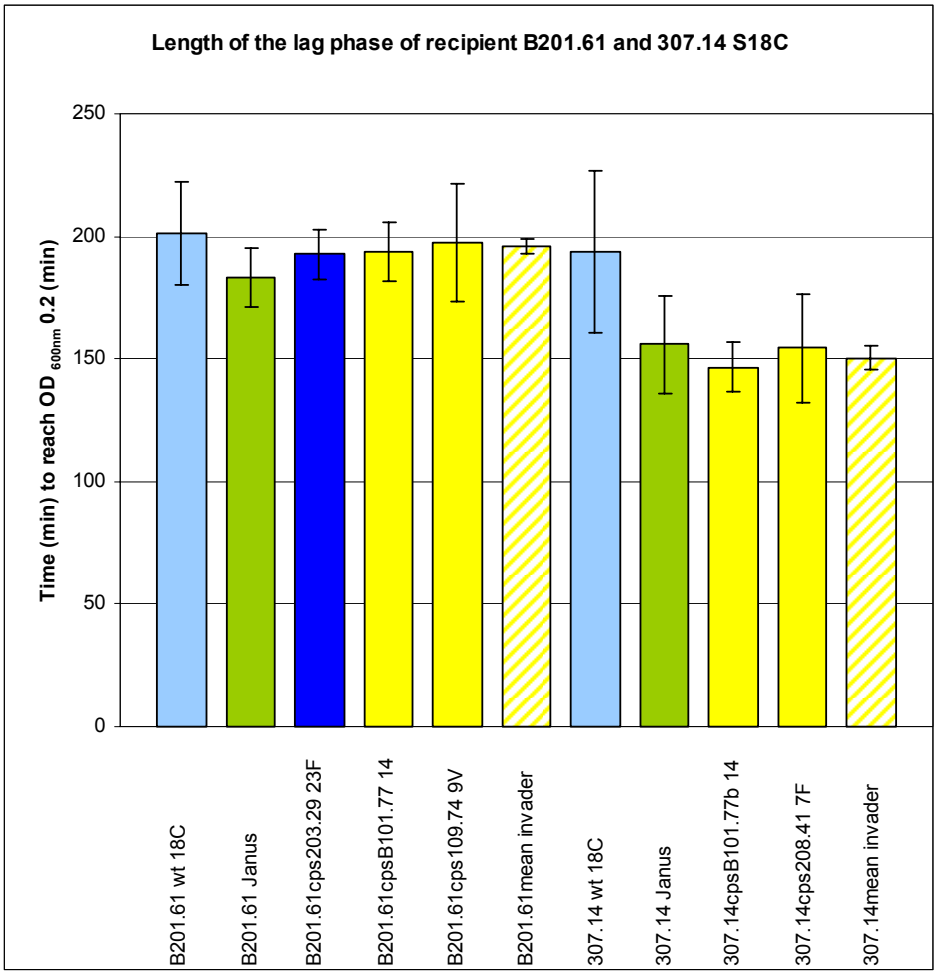


Fig. A7 Length of the lag phase of recipient B201.73 serotype 19F

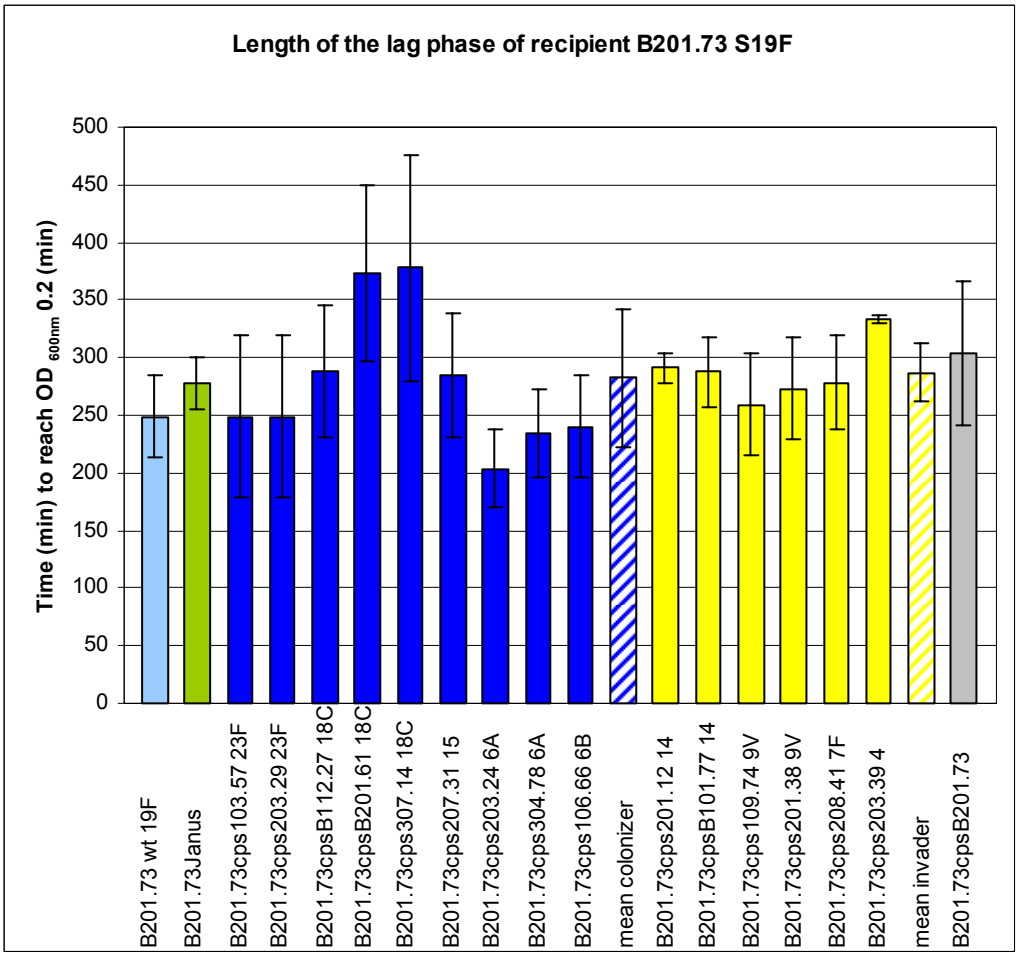


Fig. A8. Length of the lag phase of recipient 103.57 serotype 23F

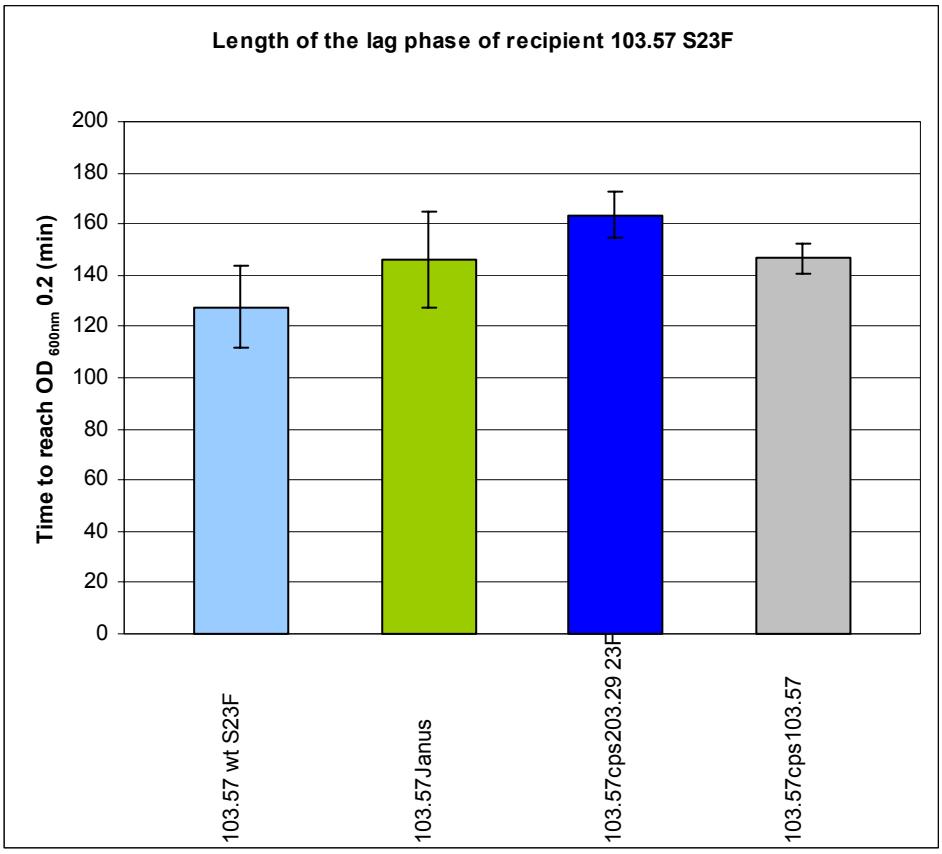


Fig. A9. Length of the lag phase of recipient B101.38 serotype 5

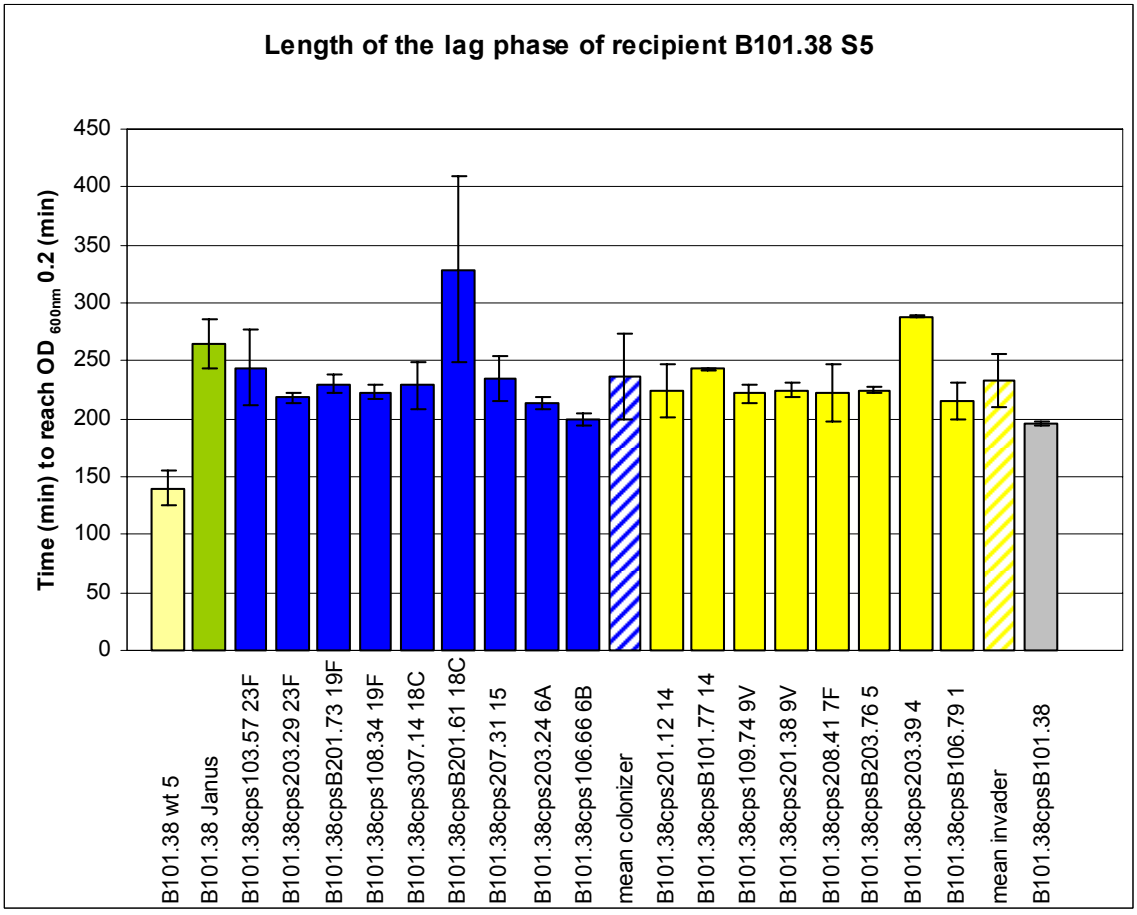


Fig. A10. Length of the lag phase of recipient 208.41 serotype 7F

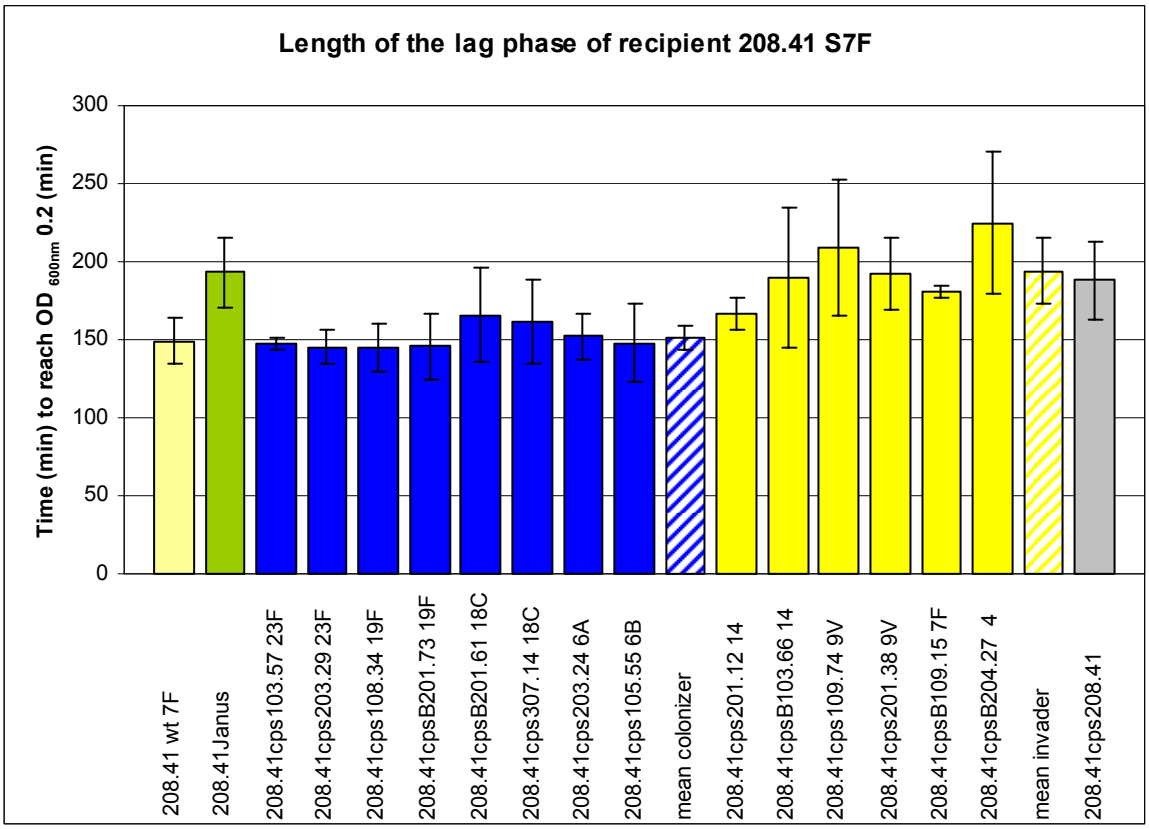


Fig. A11. Length of the lag phase of recipient B109.15 serotype 7F

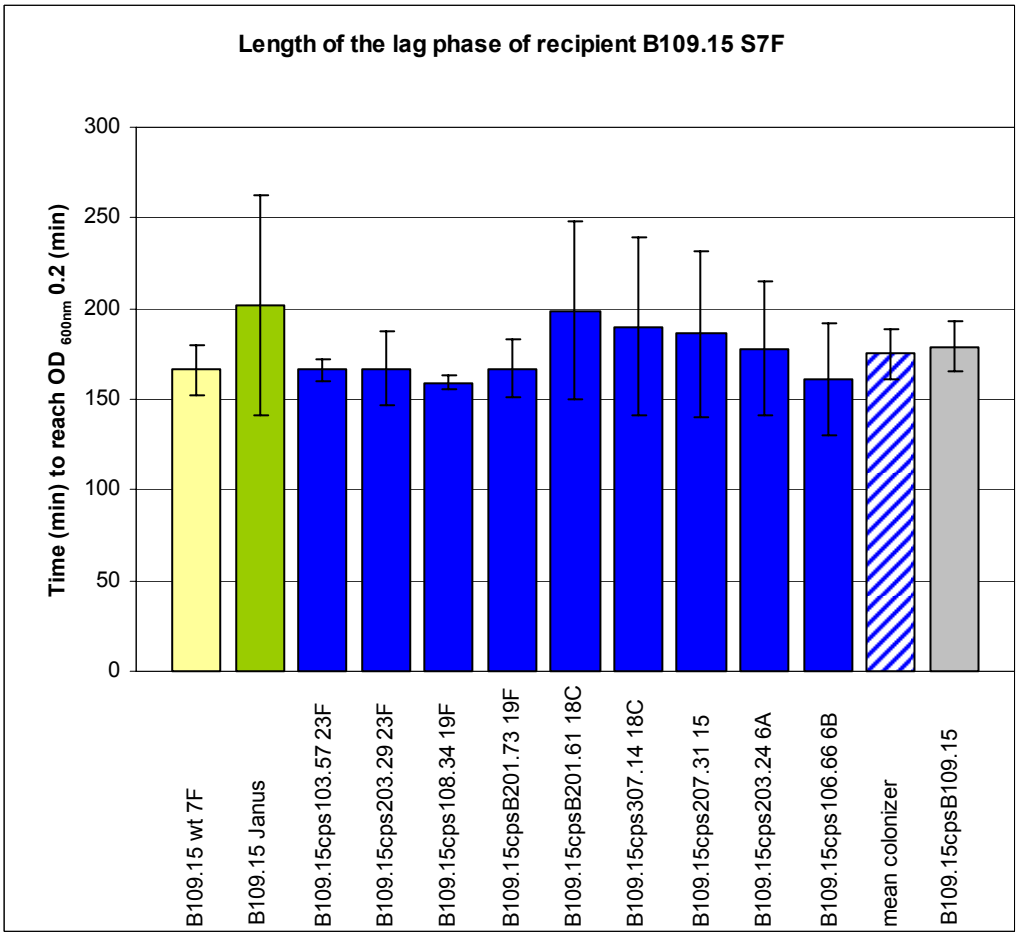


Fig. A12. Length of the lag phase of recipients B110.04 serotype 7F

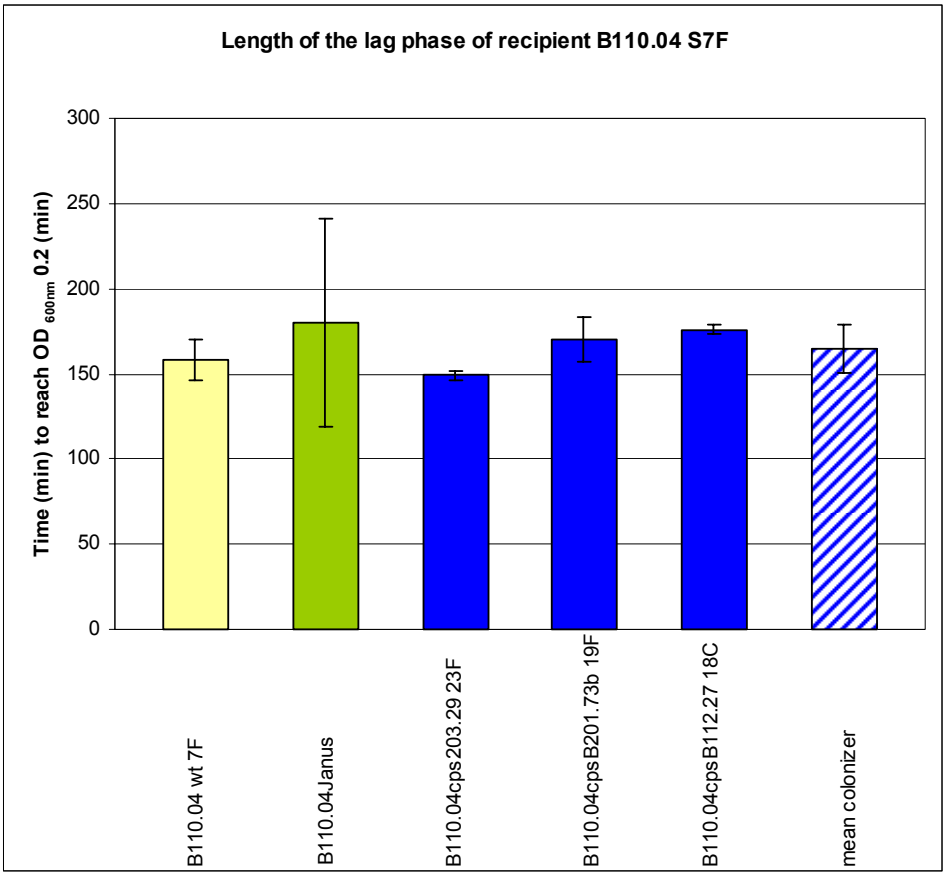
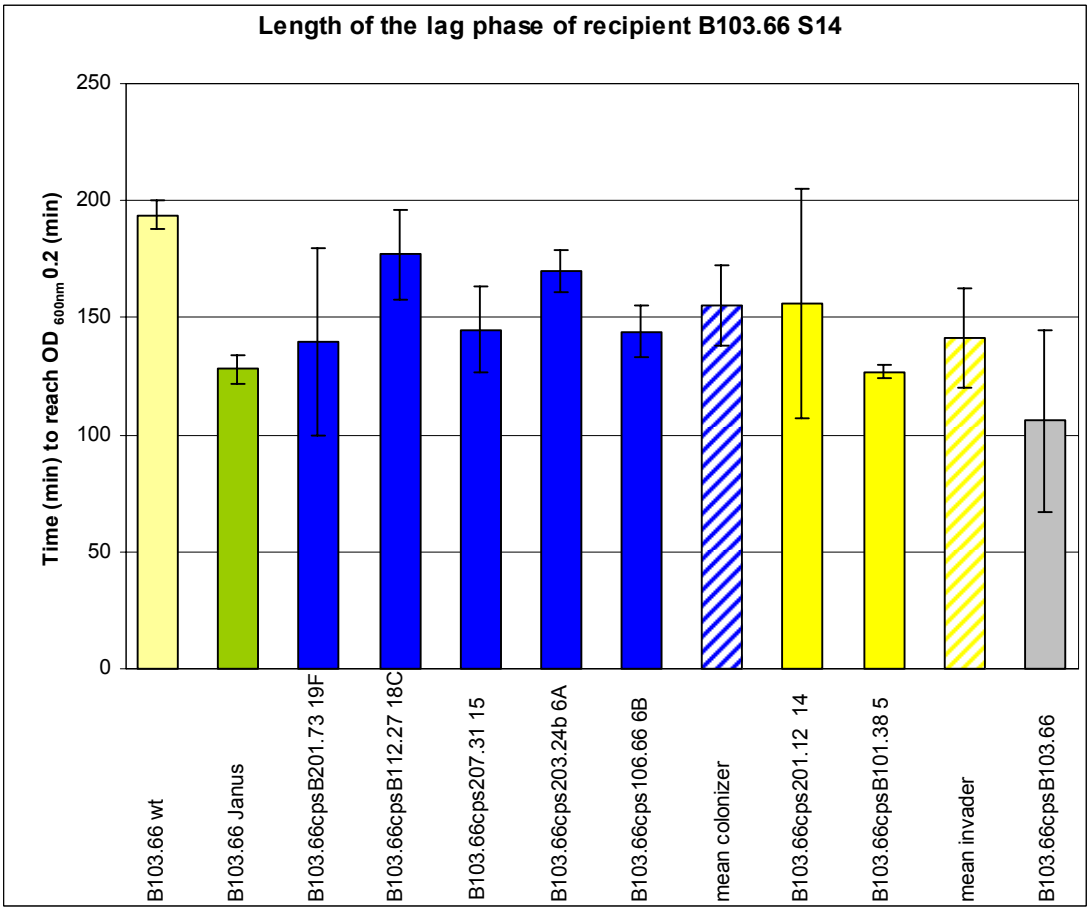


Fig. A13. Length of the lag phase of recipients B103.66 serotype 14



9. Declaration of Originality

Last name, first name: Morand Brigitte

Matriculation number: 00-113-969

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date

Bern, 05.12.2008

Signature