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Genomic analysis of β-lactam resistance mechanisms in
Streptococcus pneumoniae

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à la Faculté des études supérieures et postdoctorales de l'Université Laval
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Résumé

*Streptococcus pneumoniae* est le pathogène bactérien le plus important des voies respiratoires (pneumonie, bronchite et otite moyenne) chez les adultes et les enfants. Cette bactérie est responsable d’une morbidité et une mortalité importantes. Bien que la pénicilline présente une activité contre de nombreux isolats de *S. pneumoniae*, la résistance à cet antibiotique est aujourd'hui, fréquemment rencontrés à la fois à l'hôpital et dans la communauté. La résistance à la pénicilline chez *Streptococcus pneumoniae* est causée par un bloc de gènes codant pour des versions altérées de protéines liant la pénicilline (PLP). Néanmoins, *S. pneumoniae* a également développé des mécanismes de résistance à la pénicilline indépendants des PLPs altérées.

L'objectif principal de cette thèse était d’utiliser des approches génomiques pour comprendre le génotype et le phénotype de résistance aux ß-lactamines chez *S. pneumoniae*.

Le travail présenté dans cette thèse a indiqué que des mutations dans les PLPs ne sont pas suffisantes pour obtenir une résistance de haut niveau à la pénicilline et au céfotaxime. Cette étude a indiqué également que la sélection de la résistance à la pénicilline chez *S. pneumoniae* peut impliquer l’acquisition de mutations conférant une tolérance à l’accumulation d’oxydants causée par les antibiotiques. Cette tolérance peut se traduire par une augmentation de la survie qui permet possiblement la sélection des déterminants majeurs de résistance tels que des mutations dans les PLPs. Dans le cas des souches cliniques résistantes à la pénicilline, nous présentons également un nouveau rôle pour une alpha-amylase cytoplasmique conférant une résistance modérée à la pénicilline en présence d'altération des PLPs. Par ailleurs, nos travaux sur la résistance au céfotaxime chez *S. pneumoniae* a permis la découverte de nouveaux gènes impliquées dans la résistance au céfotaxime, y compris les gènes spr1333, spr0981, spr1704 et spr1098 qui codent respectivement pour un peptidoglycan GlcNAc déacetylase, une glycosyltransférase, un transporteur ABC et une sortase. Nos travaux génomiques ont permis de découvrir de nouveaux gènes de résistance aux ß-lactamines chez *S. pneumoniae*. 
Abstract

Streptococcus pneumoniae is the most important bacterial pathogen of the respiratory tract (pneumonitis, bronchitis and otitis media) in adults and children resulting in significant morbidity and mortality. Although penicillin shows activity against many isolates of S. pneumoniae, resistance to this antibiotic is now frequently encountered, both at the hospital and in the community. Penicillin resistance in Streptococcus pneumoniae is mediated by a mosaic of genes encoding altered penicillin-binding proteins (PBP). Nonetheless, S. pneumoniae has also developed non-PBP mechanisms implicated in penicillin resistance.

The principal objective of this thesis was to use global sequencing approaches to understand β-lactam resistance genotype and phenotype in S. pneumoniae.

The work presented in this thesis indicated that mutations in PBPs are not sufficient to achieve high level resistance to penicillin and cefotaxime. This study also indicates that the selection of resistance to penicillin in S. pneumoniae involves the acquisition of mutations conferring tolerance to the antibiotic-induced accumulation of oxidants. This tolerance can translate into an increased survival that putatively enables the selection of major resistance determinants such as mutations in PBPs. In the case of clinical isolates, we also report a new role for a cytoplasmic alpha amylase in conferring moderate resistance to penicillin in the presence of altered PBPs. Furthermore, our works on cefotaxime resistance has allowed the discovery of novel cefotaxime resistance genes in S. pneumoniae including spr1333, spr0981, spr1704 and spr1098 coding respectively for a peptidoglycan GlcNAc deacetylase, a glycosyltransferase, an ABC transporter, and a sortase were implicated in resistance to cefotaxime. Our genomic approaches were useful to discover novel β-lactam resistance genes in S. pneumoniae.
Avant-Propos

Over the years of carrying out the project detailed in the present thesis, I had the privilege of knowing wonderful individuals in the “Centre de Recherche en Infectiologie” at CHUL, without whose invaluable help this work would have never been materialized. I would therefore like to express my deeply-felt thanks to every one of them.

First and foremost, I would like to thank my supervisor, Professor Marc Ouellette, for giving me the opportunity to initiate my PhD programme in his laboratory, and for the confidence and trust he showed me during my academic career. His academic support as well as his incredible patience had a prime role in the success of this research project. Equally valuable to my academic development were his scaffolding at the start of my study, the scientific discussions he held with me on a regular basis, and his constant availability.

I would also like to thank the external reviewers of my thesis, Dr. Jacque Corbeil and Dr. Sylvain Moineau from Université Laval, and Dr. Patrick Trieu-Cuot from “Institut Pasteur de Paris”, for taking the time and trouble to provide careful and critical examinations of my thesis. I am also grateful to the director of Microbiology-Immunology program, Dr. Sylvain Bourgoin, and to Chantal Joubert, the “Agente de gestion des études”, for their collaborations during the submission process.

My special thanks would go to all the members of the team working under the supervision of Professor Marc Ouellette, the so-called “Mou Team”, whom I met over the years. I deeply appreciate their regular constructive comments on my work and for their valuable esprit de corps. I would like to thank Suzanne Avoine, the “mother of lab” as she is often called by colleagues, for her friendly support in answering my technical as well as language-related questions, not to mention the pleasant atmosphere she constantly created in the lab. Many thanks to Andréanne Lupien, Hélène Gingras and Dr. Dewan Billal, the members of Streptococcus group, for their scientific comments and their pleasant and valuable friendship. Equally thankful I am to Marie-Christine Brotherton for her help with setting up and running the SILAC experiment and with analyzing the related data, to Isabelle Girard, who gave me training on how to
work with the streptococcus, and to Danielle Legaré for her academic guidance over the first months of my work in the lab. I would also like to express my special thanks to Dr. Philippe Leprohon for his critical comments on the three research reports presented in this thesis. Without a doubt, I am indebted to “Fondation de l’Université Laval” for the financial support it provided me with over a good part of my PhD programme.

Last but definitely not the least, I would like to thank the members of my family. I appreciate all the support and encouragement I received from my mother and my sisters during my study. A very special thank would go to my dear husband, Faramarz, without whose love and sacrifices as well as his editing assistance I would not have brought this thesis to its end. Finally I would like to thank my dear and lovely son, Daniel, for his patience and constant smiles that warmed my heart on a daily basis.
Contributions

The present thesis is the result of the work carried out under the direct supervision of professor Marc Ouellette. Structurally, it compiles, among other parts, three research articles to which the author has contributed. These articles, which make up three different chapters in the thesis, are listed below.

1. “Whole genome sequencing of penicillin-resistant *Streptococcus pneumoniae* reveals mutations in penicillin-binding proteins and in a putative iron permease” was published in 2011 in *Genome Biology*. This article makes up chapter VII. My contribution included designing and performing the experiment as well as analyzing the data under the supervision of Dr. Marc Ouellette. The integrative plasmids used in this project were also generated by me. Dr. Philippe Leprohon and Dr. Danielle Légaré revised the manuscript and provided critical comments.

2. “Genome analysis and reconstruction of cefotaxime resistant in *Streptococcus pneumoniae*” was published in 2013 in *Journal of Antimicrobial Chemotherapy*. The article is presented in chapter VIII of the thesis. I was responsible for designing, performing and analyzing all of the biological experiments as well as writing up of the manuscript. Professor Marc Ouellette supervised the project and Dr. Philippe Leprohon revised the manuscript and provided critical comments.

3. “Genomic analyses of DNA transformation and penicillin resistance in *Streptococcus pneumoniae clinical isolates*”, presented in chapter IX of the thesis, was submitted to *journal of Antimicrobial Chemotherapy* for publication. Here again, I designed, performed and analyzed all the biological experiments and produced the manuscript. Dr. Marc Ouellette supervised the project and Dr. Philippe Leprohon provided critical comments on manuscript.
For my son, Daniel, and my husband, Faramarz, whose love, supports and encouragements provided the impetus for this work
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>Choline-binding protein</td>
</tr>
<tr>
<td>CDM</td>
<td>Chemically defined medium</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>CGS</td>
<td>Comparative genome sequencing</td>
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<tr>
<td>CIP</td>
<td>Ciprofloxacin</td>
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<tr>
<td>CSP</td>
<td>Competence stimulating peptide</td>
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<td>CTX</td>
<td>Cefotaxime</td>
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<td>DAP</td>
<td>Diaminopimelic acid</td>
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<td>DGH</td>
<td>Distributed genome hypothesis</td>
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<td>DHFR</td>
<td>Dihydrofolate reductase</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
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<tr>
<td>EF</td>
<td>Elongation factor</td>
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<tr>
<td>FQ</td>
<td>Fluoroquinolone</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GT</td>
<td>Glycosyltransferase</td>
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<tr>
<td>HMM</td>
<td>High molecular mass</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>IPD</td>
<td>Invasive pneumococcal disease</td>
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<tr>
<td>iTRAC</td>
<td>Isobaric tagging for relative and absolute quantification</td>
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<td>LMM</td>
<td>Low molecular mass</td>
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<td>LZD</td>
<td>Linezolid</td>
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<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MRSA</td>
<td>Meticillin-resistant staph aureus</td>
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<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NCSP</td>
<td>Non-classical surface protein</td>
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<td>PBP</td>
<td>Penicillin-binding protein</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PCV</td>
<td>Pneumococcal conjugate vaccine</td>
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<td>PG</td>
<td>Penicillin G</td>
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<td>PGN</td>
<td>Peptidoglycan</td>
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<td>PNSP</td>
<td>Penicillin non-susceptible streptococcus pneumoniae</td>
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<td>PPV</td>
<td>Pneumococcal polysaccharide vaccine</td>
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<td>PRS</td>
<td>Penicillin-resistant streptococci</td>
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<td>QRDR</td>
<td>Quinolone-resistance determining regions</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RPR</td>
<td>Ribosomal protection proteins</td>
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<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
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<td>SILAC</td>
<td>Stable isotope labeling of amino acids in cell culture</td>
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<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
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<td>ssDNA</td>
<td>Single stranded deoxyribonucleic acid</td>
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<tr>
<td>TP</td>
<td>Transpeptidase</td>
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<tr>
<td>VRE</td>
<td>Vancomycin-resistant enterococci</td>
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<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
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<tr>
<td>WHO</td>
<td>World health organization</td>
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<td>WT</td>
<td>Wild-type</td>
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Chapter I. *Streptococcus pneumoniae*

*Streptococcus pneumoniae* is a Gram-positive coccus of the order Lactobacillales, family Streptococcaceae and genus *Streptococcus*. *Streptococcus pneumoniae* (the pneumococcus) belongs to the species of streptococci which include other pathogenic bacteria such as *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus suis*, *Streptococcus uberis*, *Streptococcus bovis* and *Streptococcus mutats*. The taxonomy of *S. pneumoniae* (the pneumococcus) has evolved since separate descriptions in the saliva by Pasteur (Pasteur, 1881) and as *Micrococcus lanceolatus* by Sternberg (Sternberg, 1881). In 1903 Schottmüller described *Streptococcus mucosus* (Schottmüller, 1903) but within a couple of years it was reclassified as pneumococcus (a term used since 1886 (Fraenkel, 1886)) on the basis of its biochemistry and specific agglutination and agglutinin absorption tests despite its markedly different mucoid morphology on solid agar. From 1926 the genus Diplococcus was used until 1974 when it was changed to *Streptococcus pneumoniae*.

1.1 Bacteriology

*Streptococcus pneumoniae* cells are Gram-positive; lancet shapes cocci (Figure 1.1). They are seen as a pair of cocci (diplococci), single cell or in short chains under microscopic examination. *Streptococcus pneumoniae* are α-hemolytic (partially hemolytic), so a green zone surrounds *S. pneumoniae* colonies on blood agar plates. Individual cells are between 0.5 and 1.25 micrometers in diameter. They do not form spore and they are not motile.

*Streptococcus pneumoniae* is a fastidious bacterium and it grows best in 5% CO2. Nearly 20% of clinical isolates require fully anaerobic condition. In all cases, growth requires a source of catalase (e.g. blood) to neutralize the large amount of hydrogen peroxide produced by the bacteria. On blood agar, colonies characteristically produce a zone of alpha (green) hemolysis, which differentiates *S. pneumoniae* from group A
(beta haemolytic) streptococci but not from commensal alpha haemolytic (viridans) streptococci which are co-inhabitants of the upper respiratory tract.

Morphology of colonies varies and depends on the degree of encapsulation of *Streptococcus pneumoniae*. The heavily capsulated strains create large colonies with several millimeters in diameter, which look grey and very mucoid, while less capsulated strains usually form smaller colonies.

Figure 1.1. *Streptococcus pneumoniae* Gram-stain. Gram-stain of blood broth culture (Taken from Todar’s online textbook, http://www.textbookofbacteriology.net)
1.2 Identification

*Streptococcus pneumoniae* can be identified and distinguished from other streptococci by its production of alpha hemolysis on blood agar, bile solubility, inhibition by optochin (ethyl hydrocupereine) and catalase negativity (Musher 2005). One or more of these tests may be inconclusive and further phenotypic tests such as agglutination with anti-pneumococcal polysaccharide capsule antibodies or genotypic tests can be required to distinguish the isolates from closely related oral streptococci (Whatmore et al. 2000; Hanage et al. 2005).

1.3 Pneumococcal colonization and disease

The human nasopharynx is a major ecological niche of *S. pneumoniae* (Figure 1.2). The nasopharyngeal carriage is higher among children mainly during the first years of life (Driver 2012b). However, *S. pneumoniae* remains as a leading cause of morbidity and mortality among the young children in developing country throughout the world. The World Health Organization (WHO) estimated that 10.6 million children less than 5 years of age present with pneumococcal disease every year and that 1.4 million children die due to pneumonia (WHO, 2011).

When *S. pneumoniae* spread from nasopharynx to lower respiratory tract or other sterile sites like blood, it may cause invasive pneumococcal diseases (IPD) such as bacteremic pneumoniae, meningitis, otitis media and bacteremia (Lynch and Zhanel 2010) (Figure 1.2).

1.4 Virulence factors

The pathogenicity of the pneumococcus has been linked mainly to its surface proteins. The capsule has a protective role and allows the pneumococcus to evade phagocytosis by immune cells. Other factors, including the cell wall polysaccharide
and pneumolysin are involved mainly in the inflammation caused by infection (AlonsoDeVelasco et al. 1995).

Figure 1.2. Infections caused by *S. pneumoniae*. All pneumococcal diseases are preceded by nasopharyngeal carriage, after which the bacterium can spread to the middle ear causing otitis media or disseminate to the lungs causing pneumonia. During infection of the lungs, bacteria may penetrate into the blood circulation, from which they can spread through the whole body. Otitis media might also cause infection of the meninges.
1.4.1 Surface proteins

There are four cluster of surface proteins in pneumococcus involved in virulence: proteins with an LPxTG motif (13 in R6 and 19 in TIGR4), the lipoproteins (42 in R6 and 47 in TIGR4), the choline-binding-protein (CBP) family (10 in R6 and 15 in TIGR4) and non-classical surface proteins that lack a classical leader peptide and membrane anchoring motifs have been identified on the pneumococcal surface (Bergmann and Hammerschmidt 2006; Perez-Dorado et al. 2012). A few examples of each category with major roles in colonization and virulence are discussed below:

1.4.1.1 LPxTG containing proteins

LPxTG proteins are covalently linked to the peptidoglycan after cleavage of their LPxTG motif by a sortase (Paterson and Mitchell 2006). The two genes, nanA and nanB, that encode neuraminidase contain LPxTG anchoring motif. They cleave sialic acid from glycoproteins, glycolipids and polysaccharides on host cell surfaces. Two zinc metalloproteases including IgA1-protease and Zmp-C that cleave mucosal IgA1 antibody and extracellular matrix respectively are two other examples of the surface proteins with an LPxTG motif (Paterson and Mitchell 2006).

1.4.1.2 Choline binding proteins (CBPs)

*S. pneumoniae* can produce 13 to 16 CBPs including three cell wall hydrolase that are important for virulence including LytA, LytB and LytC. These autolysines hydrolyze murein of the cell wall (Paterson and Mitchell 2006). Interestingly autolysis induced by LytA is important for the release of major virulence factors, such as pneumolysin. CbpA, PspA and PcpA and is link to adhesion to host cells and colonization in mice.

1.4.1.3 Lipoproteins

Pneumococcal lipoproteins including PsaA, an ABC type manganese transport system, and Ami/AliAB, the oligopeptide permease have been shown to be essential for substrate transport and virulence (Kerr et al. 2004; Anderton et al. 2007).
1.4.1.4 Non classical surface proteins

Non classical surface proteins (NCSPs) are proteins lacking classical leader peptide and membrane-anchoring motifs. The enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-enolase which are involved in carbohydrate metabolism, represent non-classical surface proteins (Hammerschmidt 2006). PavA protein is another example of NCSP which has been identified as a pneumococcal adhesion protein for fibronectin and as an important factor involved in adherence, invasion and modulation of meningeal inflammation (Holmes et al. 2001; Pracht et al. 2005).

1.4.2 Capsule

The most important virulence factor of *S. pneumoniae* is its capsule. A capsule composed of polysaccharide completely covers the pneumococcal cell, interfering with phagocytosis by preventing complement C3b opsonisation of the bacterial cells. During invasion the capsule is an essential determinant of virulence. Currently at least 90 different capsule types (serotypes) of pneumococci have been recognized and form the basis of antigenic serotyping of the organism (Driver 2012b).

1.4.3 Pneumolysin

Pneumolysin, which represents a cholesterol-dependent cytolysin, is another major virulence factor in the pneumococcus. Pneumolysin has multiple biological activities; it has pore-forming activity in host cell and it can trigger the immune system (i.e. complement activation and pro-inflammatory responses (Paterson and Mitchell 2006a).

1.4.4 Cell wall

The cell wall is a layer below the polysaccharide capsule and it consists of peptidoglycan, teichoic and lipoteichoic acid and phosphorylcholine. A variety of proteins with different function are anchored to this layer. Purified peptidoglycan is a
powerful stimulus for induction of inflammation similar to that seen after infection with whole pneumococci.

1.5 Treatment

Antibiotics are the mainstay for the treatment for *Streptococcus pneumoniae* infection. Until the 1970s, all *S. pneumoniae* isolates were sensitive to all commonly used antibiotics such as penicillin, macrolides, clindamycin, cephalosporin, vancomycin and trimethoprim-sulfamethoxazole. Beginning in the 1990s, many pneumococcal isolates showed decreased susceptibility to penicillin and other commonly used antibiotics. Drug resistant *S. pneumoniae* are now frequently encountered, both at the hospital and in the community and more than 20% of clinical isolates of *S. pneumoniae* in the USA are multidrug resistant (Doern et al. 2001). The three main classes of antibiotics used for the treatment of *S. pneumoniae* are β-lactams, macrolides and fluoroquinolones (FQ).

1.6 Vaccination

In developed countries, serious disease occurs mainly in young children under two years of age and in the elderly. Growing resistance of *S. pneumoniae* to conventional antibiotics limits the treatment options, and emphasizes the urgent need for vaccines in order to prevent pneumococcal infections. Although there are more than 90 known serotypes of pneumococci, relatively few serotypes are responsible for serious diseases. Antibody to the capsular polysaccharide confers serotype specific protection against diseases. Vaccination is efficient in preventing invasive diseases (Whitney et al. 2003), but pneumococcal clones expressing capsular polysaccharides with serotypes not included in the current vaccines’ formulations survive, and the incidence of invasive diseases induced by these clones is increasing (Hicks et al. 2007). There are two types of pneumococcal vaccines: polysaccharide and conjugated vaccines.
1.6.1 Pneumococcal polysaccharide vaccine (PPV)

The first licensed pneumococcal vaccine (pneumovax 23) in 1983 contained 23 pneumococcal serotypes associated with most infections. The main limitation of polysaccharide vaccines is that they are T-cell independent antigens so they do not stimulate a significant immune response in the infants under 2 years of age. In addition, PPV does not induce immune memory and does not have any effect on nasopharyngeal carriage (Driver 2012b).

1.6.2 Pneumococcal conjugate vaccine (PCV)

In 2000, a pneumococcal 7-valent PCV (PCV7, Prevnar) was licensed in the United States. This vaccine composed of seven different pneumococcal polysaccharides conjugated to a protein carrier that induces T-cell dependent immune response. Immunized infants would generate robust immune response and immune memory, with associated reduction in carriage (Driver 2012b). Since 2010, several expanded valency PCVs have been licensed such as 13-valent Prevnar or 10-valent with three different protein carriers (Moffitt and Malley 2011).
Chapter II. Genetic diversity in *Streptococcus pneumoniae*

*S. pneumoniae* is an organism which exhibits a high degree of genomic diversity and natural populations appear to preserve genetic exchange in order to adapt to their environment. Whole genome sequencing of two strains of *S. pneumoniae* R6 (Hoskins et al. 2001) and TIGR4 (Tettelin et al. 2001) in 2001 and comparing the genomes of these two strains was a milestone in realizing the potential extent of diversity of pneumococcal genomes. The two genomes differed in size (R6 being 2Mb and TIGR4 being 2.16Mb) and differed in 10% of their genes (Bruckner et al. 2004). The sequence comparison of 17 strains of *S. pneumoniae* (Hiller et al. 2007) revealed that only 46% genes were conserved among all 17 strains with each strain’s genome containing between 21 and 32 % of noncore genes. The extensive genomic diversity data obtained from this study supports the distributed-genome hypothesis (DGH) which asserts that pathogenic bacteria possess a “supragenome” or gene pool that is much larger than the genome of any single isolate and that these pathogens generate genomic diversity by using genetic recombination and a large noncore set of genes as a resource of diversity generation (Hiller et al. 2007). Natural diversity in *S. pneumoniae* is generated by different mechanisms, and these will be discussed below.

2.1 Mechanisms of DNA exchange

Exchange of DNA among bacteria could occur by at least three different ways. In transformation, DNA released from one cell directly enters to another cell of the same species. In conjugation, the plasmids transfer DNA molecule from one cell to another, while in transduction, a bacterial virus (bacteriophage) picks up the DNA molecule from a cell it has infected and injected it to another cell.
2.1.1 Transformation (competence)

Bacterial transformation is widely distributed in nature and contributed to the adaptation and ecological diversification of several bacterial species (Johnsborg et al. 2007). Genetic transformation requires the development of competence, a genetically programmed transient state in which the bacteria has the ability to transfer the DNA to the cytosol (Berge et al. 2002). Competence development in *S. pneumoniae* is carefully controlled by a *comCDE* operon which encodes a competence-stimulating peptide (CSP), a membrane bound histidine kinase, and a cognate response regulator (Havarstein et al. 1996; Pestova et al. 1996) (Figure 2.1). Pre-mature CSP (42-amino-acid prepeptide) encoded by *comC*, is processed in parallel with its export outside the cell by the *comAB* (ABC transporter) secretion apparatus (Hui, 1991, Havarstein, 1995). The extracellular concentration of CSP is monitored and is regulated by a two-component signal transduction system, ComDE (Stock et al. 2000) (Figure 2.1). When the concentration of CSP reaches a critical level, Com D is activated, phosphorylating its cognate regulator (ComE) leading to up-regulation of the early competence gene, including the gene encoding the alternative sigma factor ComX (Lee and Morrison 1999). ComX activates a second wave of gene expression (late competence genes), which are involved in DNA uptake and recombination process (Lee and Morrison 1999).

The DNA uptake machinery consists of a transformation pseudopilus, made of the major pilin like (ComGC) and minor pilin like (ComGD, ComGE, ComGG); dsDNA receptor protein (ComEA); an endonuclease EndA; a membrane channel protein for ssDNA, ComEC; and an ATP-binding protein, ComFA (Claverys et al. 2009) (Figure 2.2). All component of the DNA uptake except EndA are encoded by genes belonging to the competence regulon (Claverys et al. 2006). The initial binding of dsDNA to the cell surface of competent cells without any base sequence preference is the first step during transformation (Dubnau 1999). Then following retraction of the pseudopilus, the exogenous DNA is allowed to traverse peptidoglycan and to be in contact with ComE. Then this receptor delivers dsDNA to EndA, leading to degradation of one strand, thus allowing transport of other strand through a transmembrane channel,
ComEC. In the last step ComFA acts as an ATP-binding protein, mediating internalization of ssDNA (Claverys et al. 2009). So, uptake of exogenous dsDNA directly produces ssDNA in competent cells with an average length of 6kb in *S. pneumoniae* (Claverys et al. 2009).

Internalized ssDNA can be integrated into the bacterial chromosome by RecA-dependent homologous recombination process. Two cytoplasmic products of late competence genes, an Ssb and RecA, have been involved in protecting ssDNA from nucleases hydrolysis and promoting its search for homologous partner (Berge et al. 2002).

![Figure 2.1. Schematic representation of competence regulation in *S. pneumoniae* (Taken from (Johnsborg et al. 2007)). The CSP-precursor, which is encoded by *comC* gene, is processed during export by ComAB transporter, resulting in extracellular accumulation of mature CSP. Global regulators such as the serine/threonine protein kinase StkP and CiaHR two-component system regulate basal transcription of *comCDE* operon. CSP binds to ComD receptor, autophosphorylating ComD which subsequently transfers a phosphoryl group to the ComE response regulator. Then activated ComE, stimulates transcription of early competence genes. ComX which is part of these genes,](image-url)
activates transcription of late competence genes encoding fratricide trigger factors CpbD and CibAB as well as the proteins involved in DNA

Figure 2.2. A DNA uptake machine. Uptake of exogenous dsDNA produces ssDNA in competent cells. Retracting of the pseudopilus, allowing dsDNA to traverse peptidoglycan (step1), thereby conveying dsDNA into contact with ComEA (step2), this latter delivers dsDNA to EndA (step3) to degrade one strand, then allowing transport of its complement through transmembrane channel, ComEC (step4). CW, cell wall; M, membrane. (Adapted from (Claverys et al. 2009))

2.1.2 Conjugation (mobile genetic elements)

The conjugative mechanism relies on exchange of mobile genetic elements (e.g. plasmids, conjugative transposons).

Conjugation-mediated chromosome transfer has been documented only in some limited species (e.g. Escherichia coli, Salmonella) (Claverys et al. 2009). The plasmids are not a common means by which diversity is introduced into the pneumococcal genome (Bruckner et al. 2004). The role of conjugation in pneumococcal diversity is also unclear (Bruckner et al. 2004). However, the acquisition and loss of plasmids by
pneumococci has been demonstrated as comparison of the genome of R6 with its progenitor strain D39, shows loss of its pDP1 plasmid (Lanie et al. 2007). Nonetheless the plasmids are used as a tool for gene inactivation, over-expression of proteins in streptococci, allowing us to functionally validate the role of genes in resistance. One of the most frequently used non-replicative plasmid in *S. pneumoniae* is pEVP3 (Claverys et al. 1995) and it was used as a high throughput system to determine gene essentiality in *S. pneumoniae* (Thanassi et al. 2002). I have generated a series of plasmids, the pFF series (Figure 2.3) that can replicate in *E. coli*, but not in streptococci because of the absence of a streptococcal origin of replication. Once integrated, their resistance markers (chloramphenicol, Cm (pFF3); kanamycin, Km (pFF6); erythromycin, Em (pFF4) are expressed through the promoter of the *S. pneumoniae amIC* gene. The pFF plasmids have the advantages of being small, of high copy number (in *E. coli*) and in having an Eam1105I site allowing TA cloning. The low-copy replicative plasmid vector pDL289 (Buckley et al. 1995) is used to over-express proteins constitutively.

![Figure 2.3. The maps of pFF3 series plasmids.](image-url)
The conjugative transposable genetic elements (transposons) such as Tn916, Tn918, Tn5030 can promote, via their transposition functions encoded by excisase and integrase genes, their intracellular transposition or transfer as singlestranded DNA (ssDNA) to a recipient cell via a cell-to-cell contact (Rice 1998). As conjugative transposons frequently carry antibiotic resistance genes, e.g. tetQ on Tn5030 (Salyers et al. 1995), tetM gene on Tn916 (Showsh and Andrews 1992) or ermB gene on Tn1545 and Tn917 (Okitsu et al. 2005), these are of great importance when considering human health threats. Moreover, Tn916 was shown to transfer even among phylogenetically distant (i.e. Gram-positive and Gram-negative) bacteria (Bertram 1991; Clewell 1995).

2.1.3 Transduction (bacteriophage)

Pneumococcal phages (pneumophages) were first isolated in 1975 from throat swabs of healthy children, by two independent groups (McDonnell et al. 1975; Tiraby et al. 1975). One year later the presence of bacteriophage has been also reported in half of pneumococci recovered from pediatric patients and one-third of isolates from adult patients (Bernheimer 1979). It has been proposed that the majority of clinical isolates of S. pneumoniae (around 75%) contain temperate bacteriophages (Ramirez et al. 1999; Romero et al. 2009) of which four have been sequenced (Lopez and Garcia 2004). While the complete genomic sequences of several pneumococcus prophages are available in GenBank, only two virulent phage have been sequenced, namely, phage Cp-1, a member of the Podoviridae family (Martin et al. 1996) and Dp-1 which belongs to the family Siphoviridae (Sabri et al. 2011). It was shown that the phages are important vehicles for the transmission of virulence genes within different species of bacterial populations (Wagner and Waldor 2002). Lytic phages appear to contribute to the natural transformation of the pneumococcus by expanding the reservoir of exogenous DNA available for incorporation into the pneumococcal genome (Ramirez et al. 1999; Lopez et al. 2000).
Chapter III. Mode of action of antibiotics and resistance mechanisms

Each year infectious diseases kill 3.5 million people (WHO, 2012). Around the middle of the 20th century, the discovery of antimicrobial agents and other means of infection control such as improved hygiene and large scale vaccination approaches helped turn the tide and for a moment human seemed to have coped with their battle against infectious microorganisms. Regarding bacterial infection, the situation dramatically improved when penicillin became available for use in the 1940s. Almost as soon as antimicrobial drugs have been used, drug resistance appeared, however. The phenomenon of antimicrobial resistance has now reached epidemic proportion. Resistant infections are more often fatal and are responsible for prolonged illness, hence increasing the likelihood of transmission to others. It has been estimated that the annual cost of antibiotic resistance infections in the USA health care system is above $20 billion.

To understand why antimicrobial drugs stop to being effective, we need first to look at their mechanisms of action (Table 3.1).

3.1 Mechanisms of action of antimicrobial agents

Most antimicrobial agents (the structure of few of them is available in Figure 3.1) may be categorized according to their principal modes of action. As summarized in Table 3.1, there are 4 major mechanisms of action: (1) interference with cell-wall biosynthesis (2) inhibition of protein synthesis (3) interference with DNA replication and repair; and (4) inhibition of a metabolic pathway.

3.1.1 Interference with cell-wall biosynthesis

The peptidoglycan (PGN) is a meshwork of strands of glycan and peptide outside the plasma membrane of bacteria, forming the bacterial cell wall. This layer confers structural strength to the cell wall (Walsh 2000). Antibacterial drugs that act on cell-wall by inhibiting peptidoglycan synthesis include the β-lactams, such as penicillins
and cephalosporins (McManus 1997). The β-lactam agents inhibit the synthesis of bacterial cell wall by interfering with transpeptidase enzymes (also termed penicillin-binding proteins or PBP s) required for the synthesis of the peptidoglycan layer (Tenover 2006). In addition to β-lactams, glycopeptides, including vancomycin and teicoplanin also target the peptidoglycan layer in the cell wall assembly. But rather than targeting PBP s, vancomycin binds to the D-alanyl-D-alanine peptide tail of the peptidoglycan precursor, preventing the incorporation of new PGN monomers into the cell wall and leads to cell death (Tenover 2006).

**Figure 3.1.** Structure of antibacterial drugs. **a**, Antibiotics include a set of natural products, penicillins, macrolides (represented by the erythromycin), tetracyclines, aminoglycosides (represented by kanamycin) and linezolid. These products inhibit protein synthesis. **b**, The fluoroquinolones (represented by ciprofloxacin) are synthetic
antibiotics, which kill bacteria by inhibiting DNA replication and repair (Taken from (Walsh 2000)).

### 3.1.2 Inhibition of protein synthesis

The RNA and protein machinery of bacterial ribosome is distinct from the analogous machinery in eukaryote cells, so there are many inhibitors of protein synthesis that selectively inhibit bacterial growth. Macrolides, aminoglycosides, tetracyclines, chloramphenicol inhibit protein synthesis by targeting different steps in ribosome action. Macrolides (erythromycin), aminoglycosides (streptomycin, kanamycin) and tetracyclines bind to the 30S ribosomal subunit, whereas chloramphenicol acts on the 50S subunit (Tenover 2006).

### 3.1.3 Interference with DNA replication and repair

The fluoroquinolones, such as ciprofloxacin are synthetic antibiotic structures that disrupt DNA synthesis and causes lethal double-strand DNA breaks during DNA replication; they do so by targeting the enzyme DNA gyrase (DNA topoisomerase type II) that is responsible for negative supercoiling of the double-stranded bacterial DNA. Quinolones antibiotics are inhibitors of DNA gyrase and act by forming a complex with the enzyme and the doubly cleaved DNA such that DNA gyrase in this complex cannot religate the cleaved DNA and lead to bacterial cell death (Walsh 2000).

### 3.1.4 Inhibition of a metabolic pathway

Antifolates including sulphonamides and trimethoprim block the pathway for folic acid synthesis. Trimethoprim is a folic acid analogue and prevents the formation of tetrahydrofolate by inhibiting the enzyme dihydrofolate reductase (DHFR), which catalyses the final stage in bacterial folate synthesis. Tetrahydrofolate is essential for purine and pyrimidine synthesis and its deficiency ultimately inhibits DNA synthesis (Sefton 2002; Tenover 2006).
Table 3.1. Targets and mode of action and mechanisms of resistance of the main classes of antibacterial drugs

<table>
<thead>
<tr>
<th>Mode of action</th>
<th>Antibiotic</th>
<th>Potential resistance mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell wall synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transpeptidases</td>
<td>β-lactams</td>
<td>β-lactamase, low affinity PBP</td>
</tr>
<tr>
<td>PBPs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Ala-D-Ala termini</td>
<td>vancomycin</td>
<td>Reprogramming of D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser</td>
</tr>
<tr>
<td>of peptidoglycan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>precursor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30S ribosomal subunit</td>
<td>Macrolides</td>
<td>rRNA methylation, drug efflux</td>
</tr>
<tr>
<td>30S ribosomal subunit</td>
<td>Tetracycline</td>
<td>Drug efflux</td>
</tr>
<tr>
<td>30S ribosomal subunit</td>
<td>Aminoglycosides</td>
<td>Enzymatic modification of drug, reduced uptake</td>
</tr>
<tr>
<td>50S ribosomal subunit</td>
<td>Chloramphenicol</td>
<td>Reduced uptake, drug inactivation, target modification</td>
</tr>
<tr>
<td>DNA replication/repair</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA topoisomerase II</td>
<td>Ciprofloxacin</td>
<td>Gyrase mutations, drug efflux</td>
</tr>
<tr>
<td>(DNA gyrase)</td>
<td></td>
<td></td>
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<tr>
<td>Folic acid synthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrofolate reductase (DHFR)</td>
<td>Trimethoprim</td>
<td>Mutations in DHFR</td>
</tr>
</tbody>
</table>

3.1.5 New paradigm for the mode of action of antibiotics

Classification of antibiotic mode-of-action is based upon drug-target interaction and upon its effect on cellular function. Antimicrobial drug targets fall into four main classes: cell-wall biosynthesis, protein synthesis, DNA replication and repair; and an essential metabolic pathway (Walsh 2000). Current antimicrobial therapies, which cover a broad range of targets (Walsh 2003), divide into two general categories: bactericidal drugs, which kill bacteria with an efficiency of more than 99.9%, and bacteriostatic drugs, which just inhibit growth (Pankey and Sabath 2004). The bactericidal antibiotic killing mechanisms have been attributed to class specific drug-target interactions, but understanding of the bacterial responses which occurs after this interaction is not complete (Tomasz 1979; Davis 1987; Lewis 2000). Recent work has revealed new mechanistic insights into how bacteria respond to bactericidal antibiotic
treatment (Dwyer et al. 2007; Kohanski et al. 2007; Kohanski et al. 2008; Davies et al. 2009; Dwyer et al. 2009; Kohanski et al. 2010). They reported that three classes of bactericidal drugs (quinolones, β-lactams and aminoglycosides) induced a common mechanism of cellular death (Kohanski et al. 2007). This study has argued that bactericidal antibiotics, regardless of their primary targets, kill bacteria by inducing alterations in iron homeostasis, ultimately leading to the accumulation of hydroxyl radicals through the Fenton reaction (Kohanski et al. 2007) (Figure 3.2). They also showed that the bacteriostatic drugs, in contrast, do not produce hydroxyl radicals. This new paradigm indicate the importance of better understanding the mechanisms by which antibiotics kill bacteria in order to find alternative antibacterial therapies to combat bacterial infections.
3.2 General mechanisms of antimicrobial drug resistance

To survive, bacteria have developed a variety of antimicrobial resistance mechanisms. There are three types of antibiotic-resistance strategies, which deal with most of the main classes of antibacterial drugs listed in Table 1. These mechanisms of resistance are shown in Figure 3.3 and are discussed in detail in the rest of this chapter.

Figure 3.2. Proposed model for the common mechanism of killing by bactericidal drugs (Kohanski et al. 2007)
The five main mechanisms that bacteria use to resist antibacterial drugs are shown in the figure. 

- **a)** The site of action (enzyme, ribosome or cell-wall precursor) can be altered.
- **b)** The inhibited steps can be bypassed.
- **c)** Bacteria can reduce the intracellular concentration of the antimicrobial agent, either by reducing membrane permeability or by active efflux of the agent.
- **d)** They can inactivate the drug. For example, some bacteria produce β-lactamase, which destroys the penicillin β-lactam ring.
- **e)** The target enzyme can be overproduced by the bacteria (Taken from Coates et al. 2002).

### 3.2.1 Reduced uptake

Antibiotic must reach their specific target and accumulate at high enough concentration to be effective, so one of the main mechanisms of resistance in bacteria is
a decreased uptake of the drug. Some strains of *Pseudomonas aeruginosa* and other non-fermenting gram-negative bacilli exhibit aminoglycoside resistance due to membrane impermeabilization. This mechanism is likely chromosomally mediated and results in cross-resistance to all aminoglycosides. The level of resistance that is seen is moderate (i.e. intermediate susceptibility) (Bryan et al. 1976; Bryan and Van den Elzen 1976; Stefani and Russo 1989; Mingeot-Leclercq et al. 1999).

### 3.2.2 Efflux of the antibiotic

Antibiotic must reach their specific target and accumulate at high enough concentration to be effective. Resistant cells take up the drug as rapidly as do sensitive ones but the drug is pumped out faster than it can diffuse in, whereby the intra-bacterial concentrations are kept low and ineffective. Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. These proteins are found in both Gram-positive and -negative bacteria as well as in eukaryotic organisms (Webber and Piddock 2003). The pumps that are not specific for one substrate, may transport a range of compounds including the antibiotics of multiple classes, so such pumps can be associated with multi-drug resistance (MDR). One of the mechanism of resistance to tetracycline in both Gram-positive and Gram-negative bacteria is over-production of some membrane proteins that act as efflux pump (Walsh 2000). In *S. pneumoniae*, active efflux has been recognized as a mechanism of resistance to macrolides (Klaassen and Mouton 2005; Wierzbowski et al. 2005), fluoroquinolones (Gill et al. 1999; Marrer et al. 2006). Efflux also implicated in the resistance of *Mycobacterium tuberculosis* to ciprofloxacin and linezolid (Escribano et al. 2007).

### 3.2.3 Inactivation of the antibacterial agents

Another strategy of resistance is destruction of the chemical structure of the drugs. Some organisms may acquire genes encoding enzymes such as β-lactamases, that hydrolyse the β-lactam ring of penicillins and cephalosporins, whereby producing the
non-functional penicilloic acid product (Philippon et al. 1989), which is useless as an antibiotic. Other antibiotic classes, such as the aminoglycosides are neutralized by enzymes which modify the antibiotic, leading to chemically modified drugs which bind poorly to the ribosomes. These modifying enzymes are classified into three major classes according to the type of modification they introduced. It includes: (1) adenylyl transferases, which add AMP moieties, (2) phosphoryl transferases, which add phosphoryl group, or (3) acetyl transferases, which acetylate the amino groups of the antibiotic (Shaw et al. 1993).

3.2.4 Modification of the target

The third strategy of resistance focuses on modifying the target. Penicillin resistance can occur not only by β-lactamase expression, but also by mutation in penicillin-binding proteins, resulting in altered PBPs with lower affinity for the drug (Spratt 1994). Erythromycin resistance in resistant bacteria can emerge by a specific mutation in 23S rRNA component of the ribosome. This modification is performed by a methyl transferase enzyme Erm and leads to decrease in affinity of erythromycin for the RNA (Bussiere et al. 1998). An additional example of target modification is used by vancomycin-resistant enterococci (VRE) to escape from vancomycin effect. In VRE the vanHAX genes encode a new pathway of enzymes that adds D-alanine and D-lactate together to produce D-Ala-D-Lac as termini of peptidoglycan instead of D-Ala-D-Ala normal termini. This modification in termini of PGN lowers the binding affinity of vancomycin by 1000-fold (Bugg et al. 1991).

3.2.5 Target amplification

When a drug is administered, it is given at a prescribed dose so the active ingredient can bind and inhibit a particular enzyme, or target. If the target is amplified over and over, it reaches a point to where the drug is not binding to enough of the substrate to be effective. Essentially, gene amplification dilutes a normal dose and in order to combat it, higher dosages are needed. Target amplification has been detected in bacteria (Musher et al. 2002; Brochet et al. 2008; Sandegren and Andersson 2009) as well as in a number of eukaryotic microorganisms in response to drug exposure, reviewed in
(Guimond et al. 2003). The 13.5-kb tandem amplification carrying the five genes required for dihydrofolate biosynthesis has been detected in *Streptococcus agalactiae* which led to both trimethoprim and sulfonamide resistance. Resistance to sulfonamide probably resulted from the increased synthesis of dihydropteroate synthase, the target of this antibiotic, whereas the amplification of the whole pathway was responsible for trimethoprim resistance (Brochet et al. 2008).

### 3.2.6 Alternative pathway metabolism

The metabolic genotype of an organism can be changed through loss and acquisition of enzyme-coding genes, allowing pathogens to evolve resistance to antimetabolites (Barve et al.). Some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acid in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid (Skold 2000; Tenover 2006).

### 3.3 Specific mechanisms of resistance in *S. pneumoniae*

Despite the availability of appropriate antimicrobial treatment, pneumococcal diseases kill 1.6 million people each year, of which one million are children under five years in developing countries (Levine, 2006). *S. pneumoniae* is the most common cause of community-acquired pneumonia, meningitis, and bacteremia in children and adults (Klugman 2001). The three main classes of antibiotic used for the treatment of *S. pneumoniae* are β-lactams, macrolides and fluoroquinolones (FQ). There are other antibiotics such as ketolides, vancomycin, trimethoprim-sulfamethoxazole, tetracyclines and linezolid (LZD) that are used for treatment of pneumococcal disease. However, drug resistant *S. pneumoniae* are now frequently encountered both at the hospital and in the community and more than 20% of clinical isolate of *S. pneumoniae* in the USA are multi-resistant (Doern et al. 2001) and I will describe resistance mechanisms to both macrolides and fluoroquinolones in this chapter as well as resistance to secondary antibiotics and devote the next full chapter to β-lactam resistance.
### 3.3.1 Macrolides resistance

Macrolide resistance in *S. pneumoniae* is due to two main mechanisms, including modification of the target or efflux pumps, which are mediated through acquisition of the *erm* or *mef* genes respectively. Acquisition of the *erm*(B) gene encoding a 23S rRNA methylase is a major resistance determinant in pneumococci. Expression of this gene reduces the affinity of macrolides for 23S rRNA by the post transcriptional modification of 23S rRNA, resulting to high level macrolide resistance (> 64 μg/ml) (Cornick and Bentley 2012). The second major mechanism of macrolide resistance in pneumococci is the acquisition of *mef* genes, encoding an active efflux pump. There are at least three subclasses of *mef* gene, of which *mef*(A) and *mef*(E) are the most abundant (Daly et al. 2004). Pneumococci carrying both *erm*(B) and *mef*(A) (referred to dual phenotype) exhibit a high level of resistance to macrolides in addition to other antimicrobials, are increasingly being described worldwide and are becoming a serious potential public health problem (de la Pedrosa et al. 2008; Reinert et al. 2008).

### 3.3.2 Fluoroquinolones (FQ) resistance

Fluoroquinolone introduced in the 1980s against Gram-negative pathogen, but increasing resistance to β-lactams and macrolides among pneumococci led to an increase usage of FQs against Gram-positive pathogens (Cornick and Bentley 2012). It has been shown that this increase in prescription led to a dramatic rise in ciprofloxacin-resistant *S. pneumoniae* in Canada (Adam et al. 2009). Resistance to fluoroquinolone in pneumococci is primarily due to accumulation of spontaneous mutations in regions known as quinolone-resistance determining regions (QRDR) of DNA gyrases (*gyrA* and *gyrB*) and DNA topoisomerases (*parC* and *parE*) (Adam et al. 2007). Most fluoroquinolone resistance ≥ 16μg/ml originates from mutations in both *parC* and *gyrA*, which confer resistance to newer fluoroquinolones (Brueggemann et al. 2002). In addition to the accumulation of spontaneous mutations, efflux pumps may also contribute to resistance to ciprofloxacin (Jumbe et al. 2006). Indeed, over-expression of the major facilitator *pmrA* (Gill et al. 1999) and of the ABC transporter *patA* and *patB* (Marrer et al. 2006) was also shown to contribute to in vitro drug resistance and also in
clinical isolates (Piddock 2006; Garvey et al. 2010). Efflux pumps alone are not known to confer high fluoroquinolone resistance $\geq 16\mu g/ml$, but their presence increases the chance of acquiring additional mutations in parC/E and gyrA/B, which confer higher level of resistance to quinolones (Jumbe et al. 2006; Piddock 2006)

### 3.3.3 Linezolid resistance

Gram positive bacteria usually develop resistance to linezolid as a result of point mutations in the gene encoding 23S rRNA (Prystowsky et al. 2001). This is the most common mechanism of resistance in staphylococci. Although resistance in clinical strains of *S. pneumoniae* has not yet been reported, a deletion in ribosomal protein L4 has been shown to lead to non-susceptibility to linezolid (Wolter et al. 2005). Other mechanisms have been identified in *S. pneumoniae* in vitro resistant isolates. These include a mutation in a 23S rRNA methyltransferase and mutations causing over-expression of the ABC proteins patA and patB (Feng et al. 2009), along with mutations in the 23S rRNA.

### 3.3.4 Tetracycline resistance

Tetracyclines are antibiotics inhibiting the bacterial growth by stopping protein synthesis. Three major mechanisms of tetracycline resistance have been identified so far: ribosome protection, efflux pumps and enzymatic inactivation of tetracycline. Ribosome protection is the most important mechanisms in tetracycline resistance in both Gram-negative and Gram-positive bacteria. This mechanism of resistance is mediated by ribosomal protection proteins such as Tet(A) and Tet(O), which shares homology with two elongation factors EF-Tu and EF-G implicated in protein synthesis. These ribosomal protection proteins (RPRs) which are called tetracycline-resistance elongation factors can protect ribosome by dislodging tetracycline from ribosome (Connell et al. 2003). Tetracycline efflux is achieved by an export protein from the major facilitator superfamily (Chopra and Roberts 2001).
3.3.5 Trimethoprim-sulfamethoxazole resistance

Trimethoprim and sulfamethoxazole are extensively used in combination as co-trimoxazole and these two components work sequentially to inhibit enzyme systems involved in the bacterial synthesis of tetrahydrofolic acid (THF) (Adrian and Klugman 1997; Schmitz et al. 2001). Resistance to co-trimoxazole in clinical isolates of *S. pneumoniae* has been reported due to mutations in dihydrofolate reductase which affect its binding to drug (Adrian and Klugman 1997; Schmitz et al. 2001).
Chapter IV. *Streptococcus pneumoniae* and β-lactam resistance

The discovery of penicillin by Alexander Fleming is unmatched with any other antibiotics, so β-lactam antibiotics, which contain a β-lactam nucleus in their molecular structure, are the most important drugs in the therapy of pneumococcal infections. Therefore, the emergence of the first penicillin-non-susceptible *S. pneumonia* (PNSP) in Australia in 1967 and rapid dissemination of penicillin resistant pneumococci is recognized as a serious public health problem (Izdebski et al. 2008).

β-lactams share the same mode of action, inhibiting synthesis of the bacterial cell wall by binding to penicillin-binding proteins (PBPs), which are involved in extracellular assembly of bacterial peptidoglycan, the essential component of the bacterial cell wall. In this chapter, we will talk about the mode of action of β-lactams by first focusing on peptidoglycan synthesis to better understand the function of penicillin-binding proteins and then we will describe PBPs and non-PBPs β-lactam resistance mechanisms in *S. pneumoniae*.

4.1 Bacterial peptidoglycan

The cell wall is responsible for the bacterial shape, but also provides strength and rigidity to protect bacteria from lysis under internal osmotic pressure (Nanninga 1998). Peptidoglycan (PGN) is a main component of the bacterial cell wall, which serves as an attachment site for virulence and adhesion factors and helps bacteria in undergoing morphological transformation in response to different stresses, and its instability may lead to cell death (Holtje 1998; Nanninga 1998). Although the chemical composition of PGN is similar in both Gram-positive and Gram-negative bacteria, the PGN layer is thinner and less cross-linked in Gram-negative bacteria.

Peptidoglycan, also known as a murein layer, is a heteropolymer composing of β-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) polysaccharide chains (glycan), which cross-linked through short peptides chain (Figure 4.1) (Rogers HJ 1980; Barreteau et al. 2008). Attached to a MurNAc residue is
a peptide chain of 4 amino acids which has the consensus sequence (L-Ala, D-glutamic acid, *meso*-diaminopimelic acid (DAP) and D-Ala) in many bacteria (Figure 4.1) (Gautam et al. 2011). There is some variation in the number and nature of the amino acids in the cross-linkage in different bacteria but all bacteria contain both D and L amino acids and the peptide chain is cross-linked to the peptide chain of another strand between the carboxyl group of D-Ala at position 4 and the amino group of *meso*-DAP (or L-Lys) at position 3 either directly or through a short peptide bridge (Vollmer et al. 2008), forming the 3D mesh-like layer.

![Figure 4.1. Architecture of bacterial peptidoglycan. DA – diamino acid (either *meso*-diaminopimelic acid or L-Lysine) (Taken from (Gautam et al. 2011))](image)
Peptidoglycan synthesis is a complex multistep process (Figure 4.2), which is divided in three stages (Rogers HJ 1980).

### 4.1.1 Stage I of PG biosynthesis

Stage I reactions occur in the cytoplasm by ten enzymes located in this compartment. The six Mur enzymes (named Mur A to Mur F; Figure 4.2) are responsible for the formation of uridine diphosphate UDP-MurNAc from UDP-GlcNAc precursor (Mur A and Mur B) and subsequently adding five amino acids onto the UDP- MurNAc (Mur C to Mur F). The peptide sequence may vary in different species but the two last amino acids should be D-Ala-D-Ala moiety (Macheboeuf et al. 2006), whose ligation is catalyzed by the Mur F ligase. This soluble UDP-MurNAc-pentapeptide becomes associated to the bacterial membrane in the next stage.

![Figure 4.2. Schematic representation of the bacterial peptidoglycan biosynthesis pathway (Taken from (Gautam et al. 2011)).](image-url)
4.1.2 Stage II of PG biosynthesis

In the second stage, UDP-MurNAc-pentapeptide molecule is attached to a membrane-bound lipid carrier molecule, named undecaprenol phosphate, through the action of phosphor-N-acetylmuramoyl-pentapeptide transferase, MraY (van Heijenoort 2001; Bouhss et al. 2004). The first lipid intermediate, undecaprenyl-pyrophosphoryl-Mur-NAc-pentapeptide formed in this stage is called lipid I. In the next step upon action of Mur G, an N-acetylglucosaminyl transferase, a soluble UDP-GlcNAc group is added to lipid I and leads to the formation of a second lipid intermediate, undecaprenyl-pyrophosphoryl-Mur-NAc-pentapeptide-GlcNAc (lipid II), which becomes the final monomer unit for PG polymer (van Heijenoort 2007; Bouhss et al. 2008). Lipid II is still in the cytoplasmic compartment and must be “flipped” over to the exterior of the membrane, which occur in the third step.

4.1.3 Stage III of PG biosynthesis

Lipid II is “flipped” over to the outer surface of cytoplasmic membrane by a “flippase” activity, which could involve either one or both membrane proteins FtsW and RodA (Holtje 1998; Nanninga 1998). Once in the outer side of the membrane, polymerisation of monomer units and binding of the newly made material to the pre-existing cell wall happens. This last stage of PG biosynthesis is catalysed by the membrane-associated enzymes with transpeptidase (TP) and glycosyltransferase (GT) activity which are known as penicillin-binding proteins (PBP). Glycosyltransferases catalyze the formation of linear glycan chain (van Heijenoort 2001b) and transpeptidases catalyze the cross-linking of the adjacent stem peptide (van Heijenoort 2001b).

4.2 Penicillin-binding proteins (PBP)

Penicillin-binding proteins (PBP), the membrane-associated enzymes, with transpeptidase (TP) and glycosyltransferase (GT) activity play essential roles in peptidoglycan biosynthesis. PBP have been divided in two main categories: high
molecular mass (HMM) PBPs and low molecular mass (LMM) PBPs (Goffin and Ghuysen 1998; Macheboeuf et al. 2006). The HMM PBPs, consist of several proteins that can be classified into two main categories class A and class B depending on the number of reactions a single polypeptide is able to catalyze. The C-terminal domain of both classes has transpeptidase activity and β-lactam antibiotics bind to its catalytic site. The N-terminal domain of class A is responsible for glycosyltransferase activity, while that of class B seems to play a role in cell morphogenesis (Marrec-Fairley et al. 2000). Both catalytic sites of bi-functional class A enzymes (TP and GT) are supposed to function independently (Di Guilmi et al. 2003).

Structural studies revealed that the TP active site of all PBPs harbors three conserved active motifs: SXXK with catalytic nucleophilic serine residue crucial for catalysis, S/YXN and a K/H(S/T)G (Hakenbeck et al. 2012). Transpeptidation reaction requires an enzyme to recognize the terminal D-Ala-D-Ala moiety of the stem peptide and catalyze the attack of carboxyl group of the penultimate D-Ala by the lateral amino group at position 3 of an adjacent chain. In the first step, transpeptidase enzyme binds to PGN stem peptide non-covalently followed by attack of the active site serine residue on D-Ala-D-Ala peptide bond, resulting in the formation of an acyl-enzyme intermediate, leading to the release of C-terminal D-Ala (acylation). The last step (deacetylation) consists of either a cross-link formation with the other PGN stem peptide (transpeptidation) or hydrolysis, with the release of tetra peptide (carboxypeptidation) (Sauvage et al., 2008). In S. pneumoniae, the acceptor involved in deacetylation is the amino group of a lysine residue. In transpeptidation reaction, the acceptor molecule can also be a water molecule, which will thus terminate a D-Ala-D-Ala carboxypeptidation reaction, which is catalyzed by low-molecular mass (LMM) PBPs, leading to the release of D-Ala and formation of tetrapeptide, preventing further reticulation of peptidoglycan (Goffin and Ghuysen 2002; Morlot et al. 2004).

4.3 β- lactam mode of action

For many years transpeptidases (TP) have been the principal target for β-lactam antibiotics (e.g., penicillin and cephalosporins). The activity of β-lactam antibiotics is
due to their structural similarity with D-Ala-D-Ala moiety of PGN (Figure 4.3). Upon binding to the active serine residue of TP domain, ß-lactams form stable covalent complex that results in acyl-enzymes that can only be hydrolyzed at a very low rate, thus preventing the cross-linking of stem-peptides and weakening of the PGN structure (Gautam et al. 2011; Hakenbeck et al. 2012). This powerful mechanism, which has made ß-lactam antibiotics the most widely used drug for any streptococcal infections for the past 70 years, has been challenged by the rapid dissemination of penicillin resistant *S. pneumoniae*.

**Figure 4.3.** Chemical structures of ß-lactam drugs (penicillins and cephalosporins). The inhibitory effect of ß-lactams comes from the structural similarity with D-Ala-D-Ala moiety of PGN chain. R1, R2, X and Z reflect different moieties which are associated to the central structure order to generate distinct antibiotics or second- or third-generation molecules. (adapted from (Macheboeuf et al. 2006))
4.4 Resistance mechanisms

Resistance to β-lactam antibiotics in clinical isolates of *S. pneumoniae* occurs through the acquisition of mosaic genes encoding altered PBPs. The mosaic genes encode PBP variants of lower antibiotic binding affinities and are the result of intra- and interspecies gene transfer events involving related streptococcal species (Chambers 1999; Hakenbeck et al. 1999). These mosaic genes differ by almost 20% at the DNA level or 10% amino acid changes compared with corresponding regions in genes of sensitive strains (Hakenbeck et al. 2012). The size of mosaic blocks within PBP genes frequently reflects the domain structure of PBPs, proposing that some kind of selective pressure related to protein function dictates the recombination sites (Martin et al. 1992; Sibold et al. 1994) and adjacent genes such as *ddl* upstream of *pbp2b* (Enright and Spratt 1999) and *ftsL* upstream of *pbp2x* (Hakenbeck et al. 2012) also may be affected.

4.4.1 PBPs mechanisms

*S. pneumoniae* contains six PBPs: the three class A HMM PBP1a, PBP 1b, and PBP 2a; the two class B HMM PBP2x and PBP2b, and the LMM PBP3. However, PBP1a, PBP2b, PBP2x and sometimes PBP2a were found to be altered in resistant clinical isolates (Figure 3.4). Depending on the selective drug, mutations in different PBPs and different sites might occur for example, PBP2b is not involved in resistance to cefotaxime (Hakenbeck et al. 1987; Smith and Klugman 2005; Stanhope et al. 2008). Figure 4.4 summarize the mutations of PBP2x, PBP2b and PBP1a associated with resistance on the linear scale and Figure 4.5 shows the overall structure of PBP2x.

4.4.1.1 Mutation in PBP2x

PBP2x is a primary resistance determinant for penicillin and cefotaxime. One selection step with penicillin and cefotaxime leads already to different PBP2x mutations (Laible and Hakenbeck 1987; Grebe and Hakenbeck 1996). PBP2x was the first PBP for which the structure was available (Pares et al. 1996), enabling detailed kinetic studies to assess the effect of individual mutations.
**Figure 4.4.** Penicillin-binding proteins profile of *S. pneumoniae*. A summary of all changes that have been found in different laboratory or clinical isolates are shown. The Six PBPs are marked in black. White: the low affinity variants in resistant mutants selected for resistance to piperacillin or cefotaxime in vitro and β-lactams clinical isolates; thin line: less PBP1a. PBP: penicillin-binding proteins (Taken from (Hakenbeck et al. 2012)).

Figure 4.5 shows the crystal structure of this primary determinant with the first amino acid of the conserved active site motifs: Ser337, Ser395 and Lys547. Selection in the laboratory demonstrated that most mutations did not map close to these active sites except for the mutations, T550A and Q552E adjacent to the K₅₄₇SG motif, and H394Y near the S₃₉₅SNmotif (Figure 4.6), which have been detected in clinical isolates as well (Coffey et al. 1995; Asahi et al. 1999; Nagai et al. 2002; Sanbongi et al. 2004).
The T550A substitution is an important contributor to low-level CTX resistance in laboratory mutants and clinical isolates of *S. pneumoniae* (Coffey et al. 1995; Grebe and Hakenbeck 1996; Sifaoui et al. 1996; Krauss and Hakenbeck 1997; Asahi et al. 1999; Sanbongi et al. 2004). A second substitution in T550G increases cefotaxime resistance even further (Grebe and Hakenbeck 1996). Threonine 550 is located in close proximity to the active site of PBP2x, where it is in direct contact with cephalosporins (Mouz et al. 1999), and the loss of hydrogen bonding between the threonine at position 550 and cefotaxime, resulting in decreased acetylation efficiency for CTX was shown to account for resistance (Gordon et al. 2000).

Comparison of several different mosaic PBP2x revealed two common point mutations including T338(A/G/P/S) next to S337 in one group and Q552E substitution in most cases, in which mutation in T338 were not seen (Mouz et al. 1998; Hakenbeck et al. 1999). The side chain of T338 implicated in hydrogen bonding to a hidden water molecule (Mouz et al. 1998), which is absent in PBP 2x containing amino acid substitution at T338 (Dessen et al. 2001). The structure of a mosaic PBP2x containing Q552E substitution revealed that this mutation displaces the β-strand with K547TG motif which leads to narrowing of the active site and a 15-fold reduction of the acetylation efficiency when T338A mutation was also present (Pernot et al. 2004).

G601V substitution has been shown to indirectly affect the active site of PBP2x by introducing a bulkier side chain involved in topological alterations of the catalytic cleft (Maurer et al. 2008). The F388L substitution is in the core of a hydrophobic niche close to the catalytic serine, along with the adjacent S389L change frequently observed in resistant isolates (Pernot et al. 2004) and could be involved in conformational alterations of the catalytic cleft.

The L364F, I371T, R384G, M400T, L546V, Y595F and N605T mutations also appear to be involved in resistance (Mouz et al. 1998; Asahi et al. 1999; Smith and Klugman 2005; Carapito et al. 2006). It has been shown that the substitution I371T and R384G led to instability of the SXN motif in position 395-397 (Dessen et al. 2001). This instability results in a slight displacement in S395, generating a more accessible
active site that may better accommodate alternative physiological substrates with branched stem peptides found in particular resistant clones (Dessen et al. 2001).

**Figure 4.5.** Crystal structure of PBP2x from *S. pneumoniae* R6. The positions of the three conserved active site motifs S337, S395 and K547 are shown with colored spheres. The dashed lines show the different domains. (Taken from (Hakenbeck et al. 2012))

### 4.4.1.2 Mutation in PBP2b

In laboratory mutants selected for piperacillin, PBP2b substitutions G600D at the C-terminal, G617A within the K₆₁₅TG motif (Hakenbeck et al. 1994) and T446A close to S₄₄₃SN motif (Grebe and Hakenbeck 1996) have been found.

The mutations T446 (A/S) and E476G are always found in PBP2b from clinical resistant isolates (over 90 sequences are available) (Dowson et al. 1993; Ferroni and
Berche 2001). The T446A change is the only substitutions that has been characterized biochemically (Pagliero et al. 2004), which displays a 60% reduction in penicillin affinity in vitro. Mutations T616S within K_{614}TG motif (Song et al. 2000) and V388A within the first active site motif S_{386}TMK (Kell et al. 1993) have been reported. In clinical isolates resistant to amoxicillin, a set of 10 mutations between residue 591-640 surrounding the third catalytic motif K_{614}TG appear to be responsible for high resistance to amoxicillin in this strain (du Plessis et al. 2002; Kosowska et al. 2004).

4.4.1.3 Mutation in PBP1a

PBP1a may be considered as the most important PBP clinically. Resistance mediated by PBP1a, can only be detected in the presence of a low affinity PBP2x and/or PBP2b (Hakenbeck et al. 2012).

In mosaic PBP1a (about 50 sequences are available) of resistant clinical isolates, T371A substitution or T371S within the first catalytic motif are frequently observed (Smith and Klugman 1998; Nagai et al. 2002). Reversion of this substitution reduced the resistance that PBP1a confers in addition to PBP2x and PBP2b (Smith and Klugman 1998). Moreover, the mutation of a stretch of four residues TSQF to NTGY at position 574-577, which is found in all the mosaic sequences (Smith and Klugman 1998; Job et al. 2003; Smith and Klugman 2003) and the mutation L539W (Smith and Klugman 2003) have been associated with resistance. Reversion of these substitutions decreased the additional resistance conferred by PBP1a (Smith and Klugman 2003). Altered residues at the entrance of the catalytic cleft resulting in modification of polarity and accessibility of the mutated PBP1a active site (Smith and Klugman 2003), but much remains to be learned about the detailed mechanism by which the PBP1a reactivity is reduced. Interaction of mosaic PBP1a with penicillin decreased acylation rate 8- to 164-fold, while in the case of interaction with cefotaxime this decrease is 2- to 25-fold (Job et al. 2008).
Figure 4.6. Site of mutations in PBP2x, PBP2b and PBP1a found in altered affinity to β-lactams. The transpeptidase domain is shown in black bar with numbers at both sides, indicating the positions of the first and last amino acids of the TP domain. The active site motifs are marked with triangle on top. (Taken from (Hakenbeck et al. 2012)).

4.4.1.4 Mutation in PBP2a

In comparison to other PBPs, PBP2a has a relatively low affinity for β-lactam antibiotics especially to penicillin, suggesting that PBP1a mutations are therefore
selected before mutations in PBP2a become a player in resistance development (Zhao et al. 2000).

In some cefotaxime laboratory mutants, PBP2a is absent due to premature termination of the transcript (Hakenbeck et al. 2012). An altered PBP2a was observed in some highly resistant clinical isolates, which has been only up to 3% different from sensitive one (Du Plessis et al. 2000; Sanbongi et al. 2004; Smith et al. 2005b; Carapito et al. 2006). In these clinical isolates, the T411A mutation flanking the active site Ser410 was frequently observed (Du Plessis et al. 2000; Smith et al. 2005b; Carapito et al. 2006).

4.4.1.5 Mutation in PBP3

A T242I substitution in PBP3, close to the active site motif K$_{239}$TG, has been associated with resistance in one laboratory mutant (Krauss and Hakenbeck 1997). A reduced amount of PBP3 due to mutation in the promoter region was found in one laboratory mutant resistant to cefotaxime (Selakovitch-Chenu et al. 1993).

4.4.2 Non-PBPs resistance mechanisms

To date, no β-lactamase–mediated resistance has been described in pneumococci. The mosaic PBPs are the primary resistance mechanism to β-lactam antibiotic in *S. pneumoniae*, However mutations in PBPs are not the sole contributors of resistance to β-lactams in *S. pneumoniae*, and evidences for non-PBP resistance mechanisms are available.

4.4.2.1 MurM and penicillin resistance

A functional operon for MurMN, which is involved in the synthesis of an alternative physiological substrate for PBPs, is required for the expression of PBP-mediated β-lactam resistance in pneumococci. In streptococci, MurM and MurN are responsible for the synthesis of branched precursor by catalyzing the addition of short dipeptide (serine-alanine or alanine-alanine) onto the amino group of stem peptide lysine as an inter-peptide bridge (Gautam et al. 2011). Interestingly, the “branched”
peptidoglycan seems to be associated with an increased level of drug resistance since the inactivation of \textit{murM} and \textit{murN} genes not only produce the murein with a large reduction in “branched” PG, but has reverted the resistance phenotype even in the presence of “resistant” PBPs (Filipe and Tomasz 2000; Weber et al. 2000; Smith and Klugman 2001).

### 4.4.2.2 PdgA and penicillin resistance

A missense mutation in peptidoglycan GlcNAc deacetylase (\textit{pdgA}) has been found following comparative genome sequencing (CGS) of high-level resistant transformant (Tait-Kamradt et al. 2009). This high level resistant transformant was obtained upon transformation of \textit{S. pneumoniae} R6 with chromosomal DNA derived from a penicillin-resistant clinical isolate in four selection step. The H266Y substitution has been detected in the fourth-step transformant in addition to some additional mutations in PPB2x, which were not present in the third-step transformants. The introduction of H266Y mutation by PCR transformation into the third-step transformant confers two-fold increased resistance only when additional mutations in PBP2x of the fourth-step were introduced simultaneously (Tait-Kamradt et al. 2009). In \textit{S. pneumoniae}, a significant proportion of the GlcNAc residues within the cell wall are N-deacetylated and the inactivation of \textit{pdgA} was shown to produce cells expressing fully N-acetylated glycans that are more sensitive to lysozyme (Vollmer and Tomasz 2000) in addition to be less virulent in an intra-peritoneal mouse model (Vollmer and Tomasz 2002).

### 4.4.2.3 Peptidoglycan \textit{O-acetyl} transferase and penicillin resistance

A mariner mutant of penicillin resistant \textit{S. pneumoniae} Pen6 showed a significant reduction in penicillin minimum inhibitory concentration (MIC) of this strain from 6 to 0.75μg/ml, allowing to identify a pneumococcal muramic acid \textit{O-acetylase} (Crisostomo et al. 2006). The inactivation of this gene has been shown to cause a considerable reduction of resistance in several penicillin resistant \textit{S. pneumoniae} strains (Crisostomo et al. 2006). The precise mechanism of this reduction of resistance is not clear but possibly that the cell wall precursor ligand with \textit{O-acetyl} group induce a necessary conformational changes to open active site of the altered PBPs in resistant
strain, and compete with the penicillin molecule for reversible binding to PBPs (Crisostomo et al. 2006). In other word, the lack of availability of the cell wall precursor with $O$-acetyl group (inactivation) would lead to lack of functional PBPs, inhibiting cell wall synthesis and leading to cell death (Crisostomo et al. 2006).

### 4.4.2.4 PstS and penicillin resistance

The results of proteomic analysis of the two independent penicillin resistance mutants selected from a sensitive clinical isolate, serotype 23F led to the discovery of an over-expressed PstS, a subunit of the phosphate ABC transporter (Soualhine et al. 2005). This protein was increase in both mutants, a phenotype correlated to increase in RNA expression of the entire phosphate ABC transporter operon. Inactivation of $pstS$ gene in the wild-type strain results in increased susceptibility to penicillin (Soualhine et al. 2005). By using real-time RT-PCR experiment, the $pstS$ mRNA levels have been measured in 12 clinical isolates, five sensitive and seven resistant strains. An excellent correlation has been obtained between resistance and increased expression of $pstS$. Inactivation of $pstS$ gene in one of the resistant clinical isolates led to a significant reduction in penicillin resistance (Soualhine et al. 2005). It has been suggested that the pneumococcal Pst system might also be part of signaling pathway (Novak et al. 1999) and its altered expression may affect regulatory pathways related to penicillin resistance (Soualhine et al. 2005).

### 4.4.2.5 CpoA and piperacillin resistance

In laboratory resistant mutants selected with piperacillin, mutations in a putative membrane-associated glycosyltransferase CpoA have been detected (Grebe et al. 1997). The $cpoA$ mutants showed various phenotypes, including a reduced susceptibility to piperacillin, less PBP1a, and a deficiency in growth rate, competence and stationary phase lysis (Grebe et al. 1997). Prokaryotic CpoA homologues are involved in teichoic acid or LPS biosynthesis and the *S. pneumoniae* CpoA was shown
to act as a lipid glycosyltransferase by transferring a galactose moiety to monogluicosyl-
diacylglycerol (α-MGlcDAG) to produce galactosyl-glucosyldiacylglycerol (GalGlcDAG), the main glycolipid in *S. pneumoniae* (Edman et al. 2003). It is thus possible that altered *cpoA* alleles may result in a modified synthesis of the polymer, thereby altering indirectly the property of the cell envelope and counteracting the activity of cell wall antibiotics.

### 4.4.2.6 CiaRH and cefotaxime resistance

The two component regulatory system CiaRH (competence induction and altered cefotaxime susceptibility) of *S. pneumoniae* was first identified in a screen for spontaneous *S. pneumoniae* mutants resistant to cefotaxime (Guenzi et al. 1994). A mutation T230P in *ciaH* led to an increase in cefotaxime resistance and to a block of genetic competence. Further work described that this mutation activated transcriptional regulation by the response regulator ciaR (Giammarinaro et al. 1999; Mascher et al. 2003; Halfmann et al. 2007). Hyper-activation of CiaR regulon led to a moderate increase of cefotaxime resistance and a total block of genetic competence (Muller et al. 2011). Deletion of *ciaR* led to an increase in susceptibility to a wide range of cell wall inhibitors (Mascher et al. 2006). It was shown that deletion of ciaR in combination with altered PBP2x resulted in rapid lysis, suggesting that in the absence of functional CiaRH regulatory system, the function of low affinity PBP2x is impaired (Zerfass et al. 2009). This deficiency was more critical in the presence of Thr338 mutation in altered PBP2x of laboratory mutant as compared with mosaic PBP2x of clinical isolates (Zerfass et al. 2009), suggesting that mosaic PBPs contain some compensatory mutations. So far, no mutations in *ciaH* have been reported in clinical isolates, while in the case of laboratory cefotaxime mutants these were frequently observed (Zahner et al. 1996; Zahner et al. 2002). There is evidence that CiaH is involved in virulence in an animal model, and thus selective pressure to keep functional CiaH in the case of clinical isolates seem likely (Zerfass et al. 2009; Hakenbeck et al. 2012).
4.4.2.7 StkP and penicillin resistance

Serine-threonine kinase (StkP) protein of *S. pneumoniae* is an important signal-transduction protein that regulates several pneumococcal proteins. These transmembrane proteins consist of an intracellular N-terminal domain, a transmembrane segment and extracellular C-terminal domain which contains a PASTA (penicillin-binding protein and serine threonine kinase) domain signature involved in cell wall sensing (Jones and Dyson 2006). The role of StkP in β-lactam susceptibility was evaluated in one laboratory mutant by mutational analysis (Dias et al. 2009). Inactivation of *stkP* gene led to increased susceptibility to penicillin but analysis of its genetic diversity in clinical isolates carrying different PBP alleles suggest strong conservation of this gene in clinical isolates (Dias et al. 2009). It has been shown that the StkP PASTA domains could interact with terminal portion of peptidoglycan stem peptide and with β-lactams (Maestro et al. 2011). In vitro studies have shown that GlmM, a phosphoglucosamine mutase implicated in the first stage of PGN biosynthesis, is a target of pneumococcal StkP, suggesting a role for StkP in cell wall metabolism (Novakova et al. 2005).

4.4.2.8 ClpL and penicillin resistance

Several stresses such as antibiotic, oxidative stress and DNA damage induce heat shock proteins (HSPs) in bacteria (Tran et al. 2011). In Gram-positive bacteria, DnaK, an important HSP, is associated with the cell wall (Kelly et al. 2005; Izquierdo et al. 2009) and it has been induced following inhibition of peptidoglycan biosynthesis in *Staphylococcus aureus* (Pechous et al. 2004). Following the report indicating that bacterial stress responses may affect antibiotic responses (Lee et al. 2009), another study has been done to assess the effect of ClpL, a member of HSP-100 (caseinolytic protease), in cell wall synthesis and antibiotic resistance (Tran et al. 2011). Mutants lacking *clpL* were more susceptible to penicillin and had a thinner cell wall than the parental strains while strains in which ClpL was over-expressed have shown a higher resistance to penicillin and had a thicker cell wall (Tran et al. 2011). This effect was due to the property of ClpL in stabilizing of PBP2x, in interacting with PBP2x, and in facilitating PBP2x translocation (Tran et al. 2011).
Chapter V. Studying antibiotic resistance by global genomic approaches

Antimicrobial resistance is an important clinical problem with tremendous implications in Canada and worldwide. In order to understand the biology of antimicrobial drug resistance in greater detail, to assess how microorganisms alter their genome and its expression to learn to live with a resistance genotype, drug resistance has to be studied at a global scale. Current thinking hypothesizes that the bacterium is not only altered in its ability to withstand the drug but also in its interaction with its environment. The use of global genomic approaches is required to comprehensively analyze resistance and associated compensatory mutations involved both in physiological and genetic adaptation of resistant bacteria.

5.1 Genome sequencing

The first published whole genome sequences for bacteria began to appear in the mid-1990s beginning with that of *Haemophilus influenzae* in 1995 (Fleischmann et al. 1995). As early as 1991, partial sequences of a pneumococcal genome (located on *Sma*I and *Apa*I fragments) were used to construct a map of the genome (Gasc et al. 1991).
Genomic approaches such as whole genome sequencing (WGS) of sensitive and resistant organisms has become a cost-effective strategy and is now emerging as a tool for studying the resistance mechanisms and the mode of action of antimicrobials. This was done initially by array-based comparative genome sequencing (Albert et al. 2005) but new sequencing technologies (454 Life Sciences, Illumina-GAI, ABI-SOLiD) are now revolutionizing the sequencing of microbial (and larger) genomes (Highlander et al. 2007; Howden et al. 2008; Cui et al. 2009; Feng et al. 2009).

5.1.1 Comparative genome sequencing (CGS)

One of the first used applications of microarray technology is to permit comparison of bacterial genomes and identify conserved regions and regions of diversity. The first applications of this technology to examine the pneumococcal genome proved that some genes such as hyaluronidase, autolysin or pneumolysin were highly conserved while cell surface proteins such as pneumococcal surface protein C, choline binding proteins and the trimethoprim resistance gene dihydrofolate reductase (dhfr) had variation in sequence (Hakenbeck et al. 2001). The absence of genes such as some capsular genes when comparing the R6 and TIGR4 genomes resulted in an understanding that there were significant regions of diversity in the pneumococcal genome and attempts at identifying a set of “core” genes.

The comparative genome sequencing technology developed by NimbleGen (www.nimblegen.com) , which relies on the use of tiled DNA microarray hybridizations to rapidly survey entire microbial genomes and to identify the location of SNPs, insertions, or deletions (Albert et al. 2005; Feng et al. 2009). The CGS protocol is divided into two phases (Figure 5.1). In the mutation mapping phase (phase I), DNAs of wild-type strain (reference) and of resistant mutant (test) were partially digested with DNase I and fragments were differentially labeled with fluorescent dyes and then hybridized to NimbleGen (Roche, Madison, WI) CGS oligonucleotide arrays derived from both strands of the reference genome. The locations of genomic alterations were identified by the ratios of the hybridization intensity labels of the two strains. In the targeted arrays, re-
sequencing (phase II), only the regions of the genome where alteration exist, were sequenced (Albert et al. 2005) (Figure 5.1). The CGS technique had tremendous potential for mutation/target identification but it is now replaced by next-generation sequencing.

![CGS Protocol Illustration](Taken from www.nimblegen.com)

1) The test DNA and reference DNA samples are separately cleaved to pools of low molecular weight fragments.

2) Each pool is labeled differentiately with two fluorescent dyes.

3) hybridized to a CGS whole genome oligonucleotide array.

4) The array images are extracted, and the ratios of test to reference DNA are noted.

5) Custom sequencing array is generated incorporating only base positions surrounding each peak on both DNA strands.

6) The labeled test DNA sample is hybridized to the sequencing array.

7) The sequencing image is extracted. The resulting sequences are compared to the reference sequence, and SNPs are identified.

**Figure 5.1.** CGS Protocol Illustration (Taken from www.nimblegen.com)
5.1.2 Next-generation sequencing

For the last three decades, the Sanger method has been the main and standard approach for DNA sequencing. The introduction of the first massively parallel pyrosequencing platform in 2005 (Margulies et al. 2005), led to a new age of high-throughput genomic sequencing now referred to as next-generation sequencing (NGS). In this section we will talk about two NGS platforms used in this study (Roche/454 life sciences and Illumina/Solexa).

5.1.2.1 Roche/454 life sciences

Whole genome sequencing by GS FLX System, gives comprehensive genome coverage and explores the full range of genetic variation using long, high-quality reads, performs straightforward de novo assembly to decode previously uncharacterized genomes, or re-sequences organisms with an available reference genome. This technology was used extensively in the analysis of vancomycin resistance in MRSA (Highlander et al. 2007; Mwangi et al. 2007; Howden et al. 2008; Cui et al. 2009), linezolid (Feng et al. 2009) in S. pneumoniae and of miltefosine resistance in Leishmania (Coelho et al. 2012).

The highly parallel sequencing system uses a novel 60×60 mm² fiber optic slide containing 1,600,000 individual wells and is able to sequence 25 million bases, at 99% or better accuracy (phred 20), in a 4 hour run (Margulies et al. 2005). There are two principal steps in massively parallel sequencing by 454 Life Sciences (Figure 5.2): Emulsion based sample preparation and sequencing in fabricated picoliter sized reaction vessels. Briefly, in the first step, the entire genome is randomly fragmented by a nebulizer and then adaptors, biotin containing primer sequences, are added to each end of the DNA fragments and the individual fragments are captured on their own streptavidin beads and within the droplets of an emulsion, the individual fragment was clonally amplified in 10 million copies. Then sequencing is performed by synthesis simultaneously in open wells of fiber optic slide using a modified pyrosequencing protocol. Nucleotide incorporation is detected by the associated release of inorganic
pyrophosphate (ppi) and the generation of photons which is detected by a CCD sensor at the bottom of each individual well (Margulies et al. 2005).

Figure 5.2. GS-FLX-system. Template DNA is fragmented, end-repaired, ligated to adapters separated into single strands and clonally amplified by emulsion PCR. After amplification, the beads are deposited into picotiter-plate wells with sequencing enzymes. The picotiter plate functions as a flow cell where iterative pyrosequencing is performed. A nucleotide-incorporation event results in pyrophosphate (PPi) release and well-localized luminescence. APS, adenosine 5’-phosphosulfate (Taken from Voelkerding et al. 2009)).
5.1.2.2 Illumina/Solexa

By 2006, the Solexa genome analyser, the first ‘short read’ sequencing platform was commercially available (http://www.Illumina.com). This technology (Figure 5.3) uses a proprietary reversible terminator-based method that enables detection of single bases as they are integrated into growing DNA strands. A fluorescently-labeled terminator is imaged as each dNTP is incorporated and then cleaved to allow incorporation of the next base. Since all four reversible terminator-bound dNTPs are present during each sequencing cycle, natural competition minimizes incorporation bias. The end result is true base-by-base sequencing that enables the industry’s most accurate data for a broad range of applications.
Figure 5.3. Illumina genome analyzer sequencing. Adapter-modified, single-stranded DNA is added to the flow cell and immobilized by hybridization. Bridge amplification generates clonally amplified clusters. Clusters are denatured and cleaved; sequencing is initiated with addition of primer, polymerase (POL) and 4 reversible dye terminators. Post-incorporation fluorescence is recorded. The fluor and block are removed before the next synthesis cycle (Taken from (Voelkerding et al. 2009)).

5.2 Transcriptomic techniques

Resistance can be mediated by point mutations but also by increased RNA and increase protein expression. The microarrays will be used in the hope of finding genes that are differentially expressed upon resistance and that may not have been pinpointed or suggested easily through the CGS analysis. DNA microarrays are used to measure the expression level of large numbers of the genes simultaneously.

DNA microarrays are created by robotic machines that arrange minuscule amounts of hundreds or thousands of gene sequences on a single microscope slide and then these slides are used for competitive hybridization of complementary DNAs of resistant and sensitive strains, which have been labeled with two different fluorescent dyes (Figure 5.4). The labeled cDNAs that represent mRNAs in the cell will then hybridize to their synthetic complementary DNAs attached on the microarray slide, leaving its fluorescent tag. Fluorescent dyes commonly used for cDNA labeling include Cy3, which has a fluorescence emission wavelength of 570 nm (corresponding to the green part of the light spectrum), and Cy5 with a fluorescence emission wavelength of 670 nm (corresponding to the red part of the light spectrum). The two Cy-labeled cDNA samples are mixed and hybridized to a single microarray that is then scanned in a microarray scanner to visualize fluorescence of the two fluorophores after excitation with a laser beam of a defined wavelength. Relative intensities of each fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes. The probes in each spot could be oligonucleotides, cDNA, or small fragments of PCR products that correspond to mRNAs.

Microarray expression experiments have been used extensively for looking at gene expression changes triggered by exposure to antimicrobial drugs (Wilson et al. 1999; Gmuender et al. 2001; Ng et al. 2003). The DNA microarray analysis have been used in
*S. pneumoniae* to detect bacterial genes conferring resistance to different antibiotics (Haas et al. 2004; Cassone et al. 2006; Marrer et al. 2006). A number of resistance markers have also been highlighted in *S. pneumoniae* by transcriptomics, including the transcriptional signatures of four different classes of translations inhibitors (Ng et al. 2003), the increased expression of putative efflux proteins in fluoroquinolone-resistant *S. pneumoniae* (Marrer et al. 2006), and the genes implicated in sugar metabolism in linezolid resistance in *S. pneumoniae* (Feng et al. 2011). In pneumococcus, microarray allowed to do comprehensive analysis of virulence, for example, comparative gene expression study revealed that the late log or early stationary phase of growth are most virulent phase of pneumococcal growth (Ko et al. 2006). Microarrays based on the human genome have also been used to investigate the host response to pneumococcal virulence factors such as pneumolysin (McDaniel et al. 2004).

**Figure 5.4.** Schematic presentation of microarray technology (Taken from http://www.genome.gov)
Use of NGS has also expanded to the field of transcriptomic. Recently, new powerful approach, termed “RNA-Seq” which sequence cDNA has replaced microarray technology. RNA-Seq has shown some advantages over gene expression array (Wang et al. 2009). Since RNA-Seq does not depend on tiling-existing genome sequence (limitation of arrays technique), allows characterization of transcription without prior information of the genome sites of transcription origin (Voelkerding et al. 2009). This technique has been rapidly adopted in a variety of organisms (Morin et al. 2008; Nagalakshmi et al. 2008; Wilhelm et al. 2008; Wang et al. 2009; Croucher et al. 2011; McNulty et al. 2011; Pichon et al. 2012) With deep coverage and base-level resolution provides information on differential expression of genes, including gene alleles and differently spliced transcripts; non-coding RNAs; post-transcriptional mutations or editing (Voelkerding et al. 2009).

5.3 Proteomic techniques

It has been shown that the correlation between RNA levels and protein expression in *S. pneumoniae*, as with other organisms, is imperfect (Soualhine et al. 2005). For example a choline binding protein was increased in penicillin resistant mutant but its RNA was down regulated (Soualhine et al. 2005). Thus a proteomic approach can be used as a complement to RNA expression profiling for analyzing changes associated with resistance.

5.3.1 Two-dimensional gel electrophoresis (2D)

Two dimensional (2D) gel electrophoresis is an established technique separating thousands of proteins in a mixture in two different dimensions. In the first dimension, the molecules are separated according to their isoelectric point. Thereby, a gradient of pH is applied to a gel and an electric potential is applied across the gel, then the proteins applied will move along the gel and will accumulate at their isoelectric point. Then the proteins are separated in the second dimension according to their molecular weight (mass). The result of this is a gel with proteins spread out on its surface, which
can then be detected by a variety of means. This method is used in combination with MS/MS to identify novel proteins involved in resistance (Drummelsmith et al. 2003; Soualhine et al. 2005).

5.3.2 Isobaric tagging for relative and absolute quantification (iTRAQ)

An alternative proteomic technique which increase proteome representation compare to 2D gel electrophoresis is iTRAQ, in which almost all peptides are labeled after trypsin digestion with isobaric tags and analyzed using multiple MS runs, thus, multiple peptides per protein are identified and quantified. iTRAQ labeling on purified cellular fractions has been used to compare the proteins of sensitive and vancomycin-intermediate resistant *Staphylococcus aureus* (Drummelsmith et al. 2007).

5.3.3 Stable isotope labeling by amino acids in cell culture (SILAC)

SILAC is a simple and straightforward approach for in vivo incorporation of a label into proteins for mass spectrometry (MS)-based quantitative proteomics. SILAC relies on metabolic incorporation of a given 'light' or 'heavy' form of the amino acid into the proteins. Thus in an experiment, two cell populations are grown in culture media that are identical except that one of them contains a 'light' and the other a 'heavy' form of a particular amino acid (e.g. 12C and 13C labeled L-lysine, respectively). When the labeled analog of an amino acid is supplied to cells in culture instead of the natural amino acid, it is incorporated into all newly synthesized proteins. After a number of cell divisions, each instance of this particular amino acid will be replaced by its isotope labeled analog.

In a recent study, the impact of PsaR-a manganese-dependent regulator- on protein expression in two strains of *S. pneumoniae*, D39 and TIGR4, has been investigated (Hendriksen et al. 2009). To do SILAC experiments, they cultured two D39 and TIGR4 wild-type strains and their PsaR mutants in chemically defined medium (CDM), supplemented with both lysine and arginine as the light or heavy isotopes. In D39Δ*psaR*, nine proteins have been up-regulated compare to wild-type. In TIGR4Δ
psaR, six proteins were more abundant and three proteins were less abundant than in the wild-type. This study revealed that PsaR of D39 and TIGR4 had a strain specific role in global gene expression and in the development of bacteremia in mice (Hendriksen et al. 2009).

Chapter VI. Rationale, Hypothesis and Objectives

Rationale

*Streptococcus pneumoniae* is the most important bacterial pathogen of the respiratory tract (pneumonitis, bronchitis and otitis media) in adults and children resulting in significant morbidity and mortality. Although penicillin shows activity against many isolates of *S. pneumoniae*, resistance to this antibiotic is now frequently encountered, both at the hospital and in the community. Penicillin resistant Streptococci (PRS) is one of the most pressing problems in antimicrobial resistance as listed by the World Health Organization. Studies of resistance mechanisms permit the development of tools for the early recognition of resistance in infection, thereby preventing useless and often toxic chemotherapy, and they may pinpoint intra-cellular drug targets and defence mechanisms, allowing the development of drug analogues.

Hypothesis

Penicillin-binding proteins have been recognized as a primary mechanism of resistance for β-lactams. Nonetheless, mutations in PBPs are not the sole contributor of
resistance and there are evidences for the implication of non-PBPs in β-lactam resistance. Indeed, strains with identical mutated PBPs can have highly different levels of β-lactam resistance. The international spread and success of a few multiple resistant clones of *S. pneumoniae* suggests that compensatory mechanisms other than resistance genes can contribute to dissemination and to the increase of the prevalence of resistance in several countries. Considerable work has dealt with the primary mechanisms of resistance, but only a few studies have dealt with the characterization of compensatory mutations in resistant cells. The comparative genome sequencing of sensitive and resistant strains proposed in this study should facilitate the discovery of non-PBPs resistance mechanisms and the identification of fitness-compensatory mutation in β-lactam resistant *S. pneumoniae*.

**Objectives**

The principal objective of the projects presented in this thesis was using the global sequencing approach to understand β-lactam resistance genotype and phenotype in *S. pneumoniae*.

More specifically, the work presented in chapter 7, had 3 specific objectives including

(1) Select and characterise resistant strains of *S. pneumoniae* to penicillin, (2) perform comparative genome sequencing of sensitive and resistant strains, (3) perform biological validation of pinpointed genes linked to the resistance genotype.

In chapter 8, the specific objectives were: (1) Analysis of *S. pneumoniae* made resistant to cefotaxime by in vitro antibiotic selection. This was done by whole genome sequencing and genetic reconstruction of resistance, (2) Analysis of *S. pneumoniae* made resistant to cefotaxime by genetic transformation of whole genomic DNAs derived from laboratory resistant mutants into sensitive reference strains to pinpoint
mutations involved in the development of resistance. The resistant transformants were characterized by NGS and resistance reconstruction.

In chapter 9, the principal objective was using functional genomics approach to discover novel non-PBPs resistance genes implicated in resistance to penicillin in clinical isolates. We first reconstructed resistance in sensitive *S. pneumoniae* by whole genome transformation using genomic DNA of three highly penicillin resistant clinical isolates as donor. Then investigation of resistance mechanisms was done by whole genome sequencing of the three transformants upon resistance reconstruction.
Chapter VII. Whole genome sequencing of penicillin-resistant *Streptococcus pneumoniae* reveals mutations in penicillin-binding proteins and in a putative iron permease

This chapter contains a manuscript that has been published in “Genome Biology” (2011, 12:R115) as a research article.

7.1 Résumé

**Contexte:** La résistance à la pénicilline chez *Streptococcus pneumoniae* est causée par un bloc de gènes codant pour des versions altérées de protéines liant la pénicilline (PLP). Néanmoins, *S. pneumoniae* a également développé des mécanismes de résistance à la pénicilline indépendants des PLPs. Le séquençage du génome d’organismes résistants a permis de découvrir des mutations impliquées dans la résistance à la pénicilline. **Résultats:** Nous avons séquencé deux souches de *S. pneumoniae* sélectionnées pour leur résistance à la pénicilline en conditions de laboratoire. L’analyse de l’assemblage des génomes a démontré que six gènes ont été mutés dans les deux mutants. Ceux-ci comprenaient trois gènes *plp*, et trois autres gènes, y compris une perméase du fer putative, *spr1178*. La mutation non-sens dans *spr1178* a toujours eu lieu lors de la première étape du processus de sélection. Bien que les mutants aient une plus grande résistance à la pénicilline, l’introduction de versions modifiées de PLP dans une souche sensible à la pénicilline par transformation séquentielle conduit à des souches avec une augmentation de la résistance moindre que chez les mutants, ce qui suggère que d’autres gènes sont impliqués dans la résistance. L’introduction par la transformation des mutations non-récurrentes PLP n’a pas augmenté la résistance à la pénicilline, mais l’introduction de la mutation non-sens dans la perméase de fer putative *spr1178* a conduit à une accumulation réduite d’espèces oxygénées réactives suite à une exposition à la pénicilline et à d’autres antibiotiques bactéricides. **Conclusions:** Cette étude indique que la sélection de la résistance à la pénicilline de *S. pneumoniae* implique l’acquisition de mutations conférant une tolérance à l’accumulation d’oxydants causée par les antibiotiques, ce qui
se traduit par une augmentation de la survie qui permet possiblement la sélection des déterminants majeurs de résistance telles que des mutations dans les PLPs.
7.2 Article

Whole genome sequencing of penicillin-resistant *Streptococcus pneumoniae* reveals mutations in penicillin-binding proteins and in a putative iron permease

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Running title: Penicillin resistance in *Streptococcus pneumoniae*

Abstract
**Background:** Penicillin resistance in *Streptococcus pneumoniae* is mediated by a mosaic of genes encoding altered penicillin-binding proteins (PBPs). Nonetheless, *S. pneumoniae* has also developed non-PBP mechanisms implicated in penicillin resistance. In this study, whole genome sequencing of resistant organisms was used to discover mutations implicated in resistance to penicillin.

**Results:** We sequenced two *S. pneumoniae* isolates selected for resistance to penicillin *in vitro*. The analysis of the genome assemblies revealed that six genes were mutated in both mutants. These included three *pbp* genes, and three non-*pbp* genes, including a putative iron permease, spr1178. The nonsense mutation in spr1178 always occurred in the first step of the selection process. Although the mutants had increased resistance to penicillin, the introduction of altered versions of PBPs into a penicillin-susceptible strain by sequential transformation led to strains with a minimal increase in resistance, thus implicating other genes in resistance. The introduction by transformation of the non-PBP recurrent mutations did not increase penicillin resistance, but the introduction of the nonsense mutation in the putative iron permease spr1178 led to a reduced accumulation of reactive oxygen species following exposure to penicillin and to other bactericidal antibiotics as well.

**Conclusions:** This study indicates that the selection of resistance to penicillin in *S. pneumoniae* involves the acquisition of mutations conferring tolerance to the antibiotic-induced accumulation of oxidants, which translates into an increased survival that putatively enables the selection of major resistance determinants such as mutations in PBPs.
*Streptococcus pneumoniae* is an important pathogen of the respiratory tract causing community-acquired pneumonia worldwide (Klugman and Feldman 2001). It is also an etiological agent of otitis media, sepsis, and meningitis in adults and children and constitutes a significant health threat. Penicillin, a β-lactam antibiotic, has long been the mainstay against pneumococcal infections but its efficacy is threatened by the rapid dissemination of penicillin non-susceptible clones worldwide, the prevalence of which varies between countries (reviewed in (Zhanel et al. 1999; Linares et al. 2010)).

β-Lactams are bactericidal antibiotics that inhibit the synthesis of the peptidoglycan layer of bacterial cell walls by inactivating penicillin-binding proteins (PBPs), a group of membrane-associated cytoplasmic proteins involved in the assembly of peptidoglycan and whose inhibition results in growth arrest and lysis. Resistance to β-lactam antibiotics in clinical isolates of *S. pneumoniae* occurs through the acquisition of mosaic genes encoding altered PBPs. The mosaic genes encode PBP variants of lower antibiotic binding affinities and are the result of intra- and interspecies gene transfer events involving related streptococcal species (Chambers 1999; Hakenbeck et al. 1999). Although *S. pneumoniae* contains six PBPs, variants of PBP2x, PBP2b and PBP1a are considered the most relevant to penicillin resistance. Furthermore, the acquisition of low-affinity PBP2x and PBP2b variants was shown to be a prerequisite for PBP1a variants to confer high-level resistance to β-lactams (Smith and Klugman 1998; du Plessis et al. 2002). PBP2x has the highest affinity for penicillin in *S. pneumoniae* and a variety of amino acid substitutions interfering with the polarity and charge distribution in the vicinity of the active site have been implicated in poor antibiotic binding and resistance (Izdebski et al. 2008; Maurer et al. 2008).

Mutations in PBPs are not the sole contributors of resistance to β-lactams in *S. pneumoniae*, and evidence for some non-PBP resistance mechanisms is available. Indeed, the cell wall of penicillin non-susceptible isolates is often highly enriched in branched chain muropeptides, a phenomenon linked to mosaic alleles of the *murM* gene (Filipe and Tomasz 2000; Smith and Klugman 2001). Furthermore, mutations in a peptidoglycan N-acetylglucosamine deacetylase (Tait-Kamradt et al. 2009), a peptidoglycan O-acetyltransferase (Crisostomo et al. 2006), a putative
glycosyltransferase (Grebe and Hakenbeck 1996), a serine threonine kinase (Dias et al. 2009), a histidine protein kinase part of a two-component signal transducing system (Guenzi et al. 1994), or in a phosphate ABC transporter (Soualhine et al. 2005) have been implicated in resistance to β-lactams.

Global approaches such as whole genome sequencing (WGS) of antibiotic-sensitive and -resistant isolates are powerful tools that are now readily available for use in determining the mode of action of antimicrobial drugs and the mechanisms involved in resistance (Albert et al. 2005; Mwangi et al. 2007; Feng et al. 2009). We report here the WGS of two independent S. pneumoniae mutants selected for in vitro resistance to penicillin and the identification of known and new mutations involved in resistance.

Results
Selection and whole-genome sequencing of *S. pneumoniae* penicillin non-susceptible mutants

Two independent penicillin-resistant mutants of *S. pneumoniae* R6 and *S. pneumoniae* 1974 were selected by stepwise penicillin increments until they reached a final penicillin minimum inhibitory concentration (MIC) of 2 μg/ml. It has not been possible to obtain mutants resistant to higher levels. The penicillin MICs of the wild-type (WT) progenitors were 0.023 μg/ml and 0.016 μg/ml for the *S. pneumoniae* R6 and 1974 lineages, respectively. The most highly resistant isolates were named R6M1 and R6M2 or 1974M1 and 1974M2 depending on whether they were derived from the *S. pneumoniae* R6 or 1974 background, respectively. All four penicillin-resistant strains were cross resistant to the cefotaxime but remained susceptible to erythromycin, tetracycline, linezolid, kanamycin and ciprofloxacin (Table 1).

We conducted WGS of R6M1 and R6M2 in order to elucidate the genetic events associated with the penicillin-resistant phenotype. The genome of R6M1 was sequenced by using the comparative genome sequencing technology developed by NimbleGen, which relies on the use of tiled DNA microarray hybridizations to rapidly survey entire microbial genomes and to identify the location of SNPs, insertions, or deletions (Albert et al. 2005; Feng et al. 2009). The comparative genome sequencing of R6M1 and WT parent allowed the identification of 26 mutations in R6M1 (Table 2) that were further confirmed by PCR amplification and conventional DNA sequencing. The genome of R6M2 was sequenced using the massively parallel 454 Life Science (Roche) GS-FLX DNA sequencing platform, which generated a genome assembly of 28× coverage, with 97% of the reads assembled into 78 large contigs. Comparative sequence analysis of R6M2 and its R6 WT parent revealed 52 mutations (Table 2) that were confirmed by PCR amplification and conventional DNA sequencing. The mutations can also be seen as part of circular schematic maps (Additional file 1).

The WGS of R6M1 and R6M2 identified a total of 40 genes that have acquired a non-synonymous mutation in at least one of the mutants (Table 2). Of these, six genes were mutated in both mutants (Table 3). These included three PBP-encoding genes, *pbp2x, pbp2b* and *pbp1a*, in which a total of 14 missense mutations and one nonsense
mutation have been observed in R6M1 and R6M2 (Table 3). The targeted sequencing of the six common genes in the 1974M1 and 1974M2 strains identified another 14 missense substitutions in PBP2x and PBP2b and nonsense mutations in PBP1a (Table 3). The T451A and G435S amino acid substitutions in PBP2b and the R384G, V518I and Q552E substitutions in PBP2x were shared by some of the mutants derived from R6 and 1974 (Table 3). The three other non-PBP-encoding genes identified by WGS mutated in both R6 mutants (although not always at the same position) were the ABC protein PstB (spr1254), the DNA mismatch repair protein HexA (spr1888) and a hypothetical protein (spr1178). Interestingly, the analysis of targeted PCR fragments from 1974M1 and 1974M2 revealed that the same nonsense mutation occurred at position 28 of the spr1178 protein in all mutants but no mutations were seen in spr1254 and spr1888 in the 1974 mutants (Table 3).

Reconstruction of resistance by transformation of mutated PBPs

Transformation experiments of S. pneumoniae R6 WT with pbp genes amplified from either the R6M1 or R6M2 mutants were conducted to assess the contribution of the different PBP mutations to penicillin resistance. The analysis of the PBP sequences at the different levels of R6M1 and R6M2 selection (0.06, 0.125, 0.25, 0.5, 1.0 and 2.0 μg/ml penicillin G (PG)) revealed a stepwise selection of PBP mutations (Additional file 2). The analysis of the chronological appearance of PBP mutations in R6M2 revealed that the progression towards penicillin resistance began with the Q281P substitution in PBP2x followed by the T451A substitution in PBP2b. The other PBP2x mutations happened sequentially as the level of resistance to penicillin increased and the remaining PBP2b and PBP1a mutations were only selected at high concentration of penicillin (Additional file 2). The pbp2x, pbp2b and pbp1a genes were amplified from R6M1 and R6M2 genomic DNA and were sequenced to confirm the presence of the mutations described in Table 3. Because of the order of mutation appearance (Additional file 2), we introduced sequentially the R6M1 mutations by first transforming the PCR fragment for pbp2x into the recipient S. pneumoniae R6 WT. The selection of transformants with 0.03 μg/ml penicillin enabled the transfer of the three PBP2x mutations found in R6M1 (R384G, V518L, Q552E). This transformant,
named R6\textsuperscript{2x-M1}, had a penicillin MIC of 0.06 μg/ml (Table 4). In a second round of transformation, the \textit{php2b} gene from R6M1 was used as donor DNA for the transformation of the recipient R6\textsuperscript{2x-M1}. Selection with 0.06 μg/ml penicillin yielded second-level transformants that acquired the two PBP2b mutations of R6M1 (D415E, G665D) and these transformants, called R6\textsuperscript{2x2b-M1}, had a penicillin MIC of 0.125 μg/ml (Table 4). All attempts to introduce \textit{php1a} mutations failed. We used a similar approach for R6M2 but in the first level transformation we pooled the \textit{php2x, 2b} and 1\textit{a} PCR fragments derived from R6M2 that were transformed into R6WT. The selection of transformants with 0.03 μg/ml penicillin enabled the transfer of only the PBP2x mutations Q281P, A369V and R384G, but not the R426C and V518I mutations despite several attempts. The transformants, named R6\textsuperscript{2x-M2}, had a penicillin MIC of 0.06 μg/ml (Table 4). In a second round of transformation, a pool of \textit{php2b} and \textit{php1a} PCR fragments from R6M2 was used as donor DNA in the transformation of the recipient R6\textsuperscript{2x-M2}. Selection with 0.06 μg/ml penicillin yielded second-level transformants that acquired the three PBP2b mutations of R6M2 (T451A, G435S and A395V) but retained an unaltered allele of \textit{php1a}. These transformants, named R6\textsuperscript{2x2b-M2}, had a penicillin MIC of 0.125 μg/ml (Table 4). Several attempts failed to introduce the PBP1\textit{a} mutation of R6M2 into the R6\textsuperscript{2x2b-M2} line.

Given that PBP1\textit{a} variants are usually associated with high level β-lactam resistance, we assessed the role of the R6M2 \textit{php1a} point mutation by transforming the R6\textsuperscript{2x2b-M2} line with the \textit{rpsL} Janus cassette (Sung et al. 2001). This required the introduction of a mutated \textit{rpsL} gene to obtain a R6 transformed cell resistant to streptomycin (see Materials and methods). The integration of the PBP1\textit{a} Janus cassette (Additional file 3) into an R6\textsuperscript{2x2b-M2} streptomycin-resistant recipient (R6\textsuperscript{SmR, 2x2b-M2}) was selected under kanamycin pressure and yielded streptomycin-sensitive and kanamycin-resistant R6\textsuperscript{2x2b-M2, 1a:janus} transformants. The replacement of the Janus cassette from R6\textsuperscript{2x2b-M2, 1a:janus} was performed in a second round of transformation with a \textit{php1a} fragment amplified from R6M2 flanked by 3 kb of upstream and downstream regions (Additional files 3 and 4). The selection under streptomycin pressure yielded the streptomycin-resistant and kanamycin-sensitive R6\textsuperscript{SmR, 2x2b1a-M2} transformants that
acquired the \textit{pbp1a} allele of R6M2. However, although the targeted sequencing of \textit{pbp1a} in R6$^{SmR}$, 2x2b1a-M2 confirmed the acquisition of the R6M2 nonsense mutation at position 411 of the protein, this strain had penicillin resistance levels identical to those of the R6$^{2x2b-M2}$ line (MIC 0.125 $\mu$g/ml; Table 4). We tested further the role of PBP1a in resistance by introducing a wild-type copy of the gene in the penicillin-resistant mutant R6M1. The co-transformation of a \textit{pbp1a} fragment amplified from \textit{S. pneumoniae} R6 WT with a \textit{rpsL} fragment conferring streptomycin resistance into R6M1 yielded the R6M1$^{SmR,1a-wt}$ transformant that harbored a WT \textit{pbp1a} allele. Interestingly, the R6M1$^{SmR, 1a-wt}$ transformant became two times more sensitive to penicillin (PG MIC 1.0 $\mu$g/ml) than its parent (Table 4).

\textbf{The inactivation of spr1178 confers tolerance to antibiotic-induced oxidants}

In addition to the mutations in \textit{pbps}, the WGS highlighted three genes that were mutated in both the R6M1 and R6M2 mutants (spr1178, spr1254 and spr1888) but only spr1178 was also mutated in 1974M1 and 1974M2. Although an increased expression of the PstB ABC transporter subunit (spr1254) had previously been associated with penicillin resistance (Soualhine et al. 2005), its inactivation by insertional duplication mutagenesis in \textit{S. pneumoniae} R6 WT did not translate into increased penicillin tolerance (Table 4). Similarly, the independent inactivation of spr1178 or spr1888 in a WT background had no effect on the level of penicillin susceptibility (Table 4). Given that the nonsense mutation in spr1178 had been acquired in every penicillin-resistant mutant analyzed, we tested whether this recurrence required a background of altered PBPs in order to confer resistance. Again, neither the independent transformation of the mutated versions of spr1178 and spr1254 into R6$^{SmR, 2x2b1a-M2}$ and R6$^{2x2b-M2}$ recipients nor the simultaneous transformation of the spr1178 and spr1254 mutations into an R6$^{2x2b-M2}$ recipient altered the penicillin susceptibility of the transformants (Table 4).

spr1178 encodes a protein of 192 amino acids with one predicted transmembrane domain that was categorized as part of the DUF3347 family of functionally uncharacterized proteins by Pfam analysis. However, a BLAST analysis for spr1178 homologues in different \textit{Streptococcus} species revealed several proteins annotated as Fe$^{2+}$/Pb$^{2+}$ permeases with at least 70% identity. Indeed, spr1178 had 86.9% and 86.7%
similarity with a putative iron permease of the FTR1 family (Stearman et al. 1996) from *Streptococcus gordonii* (Vickerman et al. 2007) and *Streptococcus mitis* ATCC6249, respectively, and 94% identity with a putative high-affinity Fe$^{2+}$/Pb$^{2+}$ permease from *S. pneumoniae* 670-6B, *S. pneumoniae* CDC3059-06 and *S. pneumoniae* P1031. Intriguingly, the bactericidal activity of antibiotics like β-lactams has recently been linked to the iron-dependent accumulation of reactive oxygen species (ROS) (Kohanski et al. 2007). Since spr1178 has a predicted iron permease function, we sought to determine whether the acquisition of the nonsense mutation in spr1178 could translate into decreased accumulation of ROS following exposure to penicillin.

Using the dichlorofluorescein diacetate (DCF-DA) dye, whose fluorescence intensity is proportional to the levels of ROS, we showed that sub-inhibitory concentrations of penicillin induced a greater time-dependent increase in ROS accumulation in a *S. pneumoniae* R6$^{SmR}$, 2x2b1a-M2 background than in cells in which the spr1178 nonsense mutation was introduced (Figure 1a). Similarly, penicillin induced a greater accumulation of ROS in R6 WT in comparison to a R6 WT strain in which spr1178 was disrupted (data not shown). We next tested whether this was a more general feature of bactericidal antibiotics and we found that ciprofloxacin (Figure 1b) and kanamycin (Figure 1c) also induced significantly more ROS in cells harboring an unaltered spr1178 gene. The time-dependent accumulation of ROS was a specific feature of bactericidal antibiotics, since the bacteriostatic antibiotics chloramphenicol (Figure 1d) and tetracycline (Figure 1e), respectively, failed to induce ROS even in the presence of a WT version of spr1178 and only induced a slight accumulation of ROS that was not correlated to the functional status of spr1178.

Growth kinetics revealed that penicillin resistance conferred a fitness cost to R6M1 (Figure 2b) and R6M2 (data not shown). The acquisition of PBP2x, 2b and 1a mutations was not associated with this growth defect, as the growth of the R6$^{SmR}$, 2x2b1a-M2 transformant was not altered compared to *S. pneumoniae* R6 WT (Figure 2a). In contrast, the introduction of a nonsense mutation in spr1178 conferred a fitness cost, as the growth of the R6$^{SmR}$, 2x2b1a, spr1178-M2 transformant was altered compared to its R6$^{SmR}$, 2x2b1a-M2 parent or to *S. pneumoniae* R6 WT (Figure 2a). Similarly, the introduction of a
WT spr1178 allele restored the fitness of R6M1, although the growth defect could not be completely reverted (Figure 2b).

**Discussion**

Whole genome sequencing of sensitive and resistant organisms is a powerful tool for understanding the biology of resistance mechanisms (Highlander et al. 2007; Mwangi et al. 2007; Howden et al. 2008; Cui et al. 2009; Feng et al. 2009). We sequenced two independent mutants selected for penicillin resistance *in vitro* to concentrate on recurrent mutations, a strategy proven to be useful (Feng et al. 2009). Resistance to β-lactams in *S. pneumoniae* clinical isolates was shown to be a complex process involving the acquisition of PBP variants of low antibiotic affinity by intra- and interspecies gene transfer events from related streptococcal species (Chambers 1999; Hakenbeck et al. 1999). Most PBP mutations directly involved in resistance were shown to alter the polarity and charge distribution around the catalytic cleft of the proteins. Indeed, the G552E substitution located in the vicinity of the active site of PBP2x (Chesnel et al. 2002) is a major determinant of β-lactam resistance (Mouz et al. 1998; Mouz et al. 1999; Pernot et al. 2004) by inducing a decreased acylation efficiency to the protein (Mouz et al. 1999). Interestingly, several PBP2x mutations have been selected in our R6 and 1974 penicillin-resistant mutants, with every resistant strain except for R6M2 having acquired the Q552E substitution (Table 3). Other PBP2x mutations that have been specifically selected in at least one of our mutants include the A369V substitution in R6M2 that was previously reported to be one of the six PBP2x mutations responsible for the β-lactam resistance of *S. pneumoniae* clinical isolates (Smith and Klugman 2005) and the G601V substitution observed in 1974M1 that was shown to indirectly affect the active site of PBP2x by introducing a bulkier side chain involved in topological alterations of the catalytic cleft (Maurer et al. 2008). The F388L substitution selected in 1974M1 was shown to be one of the three substitutions responsible for cefotaxime resistance in a *S. pneumoniae* clinical isolate (Maurer et al. 2008). The F388L substitution is in the core of a hydrophobic niche close
to the catalytic serine, along with the adjacent S389L change frequently observed in resistant isolates (Pernot et al. 2004), and could be involved in conformational alterations of the catalytic cleft. Finally, other PBP2x mutations identified in our penicillin-resistant strains probably have more indirect roles in resistance, like the R384G and R426C substitutions selected in both R6 mutants. These substitutions have also been found in a previously described series of laboratory-derived cefotaxime-resistant mutants (Laible and Hakenbeck 1987).

Reconstruction of resistance by the stepwise introduction of PBP mutations into a R6 penicillin susceptible background revealed an ordered appearance of mutations first in \textit{pbp2x}, then in \textit{pbp2b} and finally in \textit{pbp1a}. Although the R426C and V518I substitutions in PBP2x only appeared at the third and fifth level of R6M2 selection (Additional file 2), transformation experiments failed at introducing these PBP2x substitutions. The R426C substitution was previously suggested to act as a compensatory mutation that requires a specific genetic background in order to be effective (Maurer et al. 2008), which could provide a plausible explanation to our failure to transfer this mutation into the R6^{2x-M2} transformant. PBP1a variants have been previously shown to confer high-level penicillin resistance only in the presence of low affinity PBP2x (Munoz et al. 1992; Reichmann et al. 1996) and/or PBP2b (Reichmann et al. 1996). In our study, however, the introduction of the PBP1a nonsense mutation from R6M2 into the R6^{2x2b-M2} line failed to increase the level of resistance to penicillin. It also appeared that a specific PBP-unrelated genetic background is required for PBP1a to participate in resistance as the reversion of its mutation in R6M1 resulted in a twofold decrease in resistance (Table 4).

In previously characterized laboratory-derived penicillin and cefotaxime-resistant mutants (Hakenbeck et al. 1994), PBP variants associated with resistance occurred late during the selection process (Krauss et al. 1996; Rogers et al. 2007), suggesting that the initial increase in resistance during the first steps of selection involves non-PBP mutations. Similarly, the transfer of R6M2 PBP mutations to a penicillin-susceptible strain did not allow it to reach the resistance level of the parent mutant R6M2. Together, this implies that other mutations are probably involved in resistance. The
analysis for recurrent mutations in our panel of resistant strains pinpointed a nonsense mutation in the putative iron permease spr1178 that occurred early during the selection process, before any PBP mutations could be selected (with the exception of the Q281P substitution in R6M2) (Additional file 2). It has recently been argued that bactericidal antibiotics, regardless of their primary targets, kill bacteria by inducing alterations in iron homeostasis, ultimately leading to the accumulation of hydroxyl radicals through the Fenton reaction (Kohanski et al. 2007). Signaling events implicating the envelope stress-response and redox-responsive two-component systems were also found to be key players in triggering hydroxyl radical formation (Kohanski et al. 2008). Although iron-sulfur clusters were initially implicated as the source of iron (Dwyer et al. 2007; Kohanski et al. 2007), the inactivation of TonB in Escherichia coli revealed that exogenous iron can also be implicated in the hydroxyurea-induced accumulation of ROS (Davies et al. 2009). In this study, we have shown that three classes of bactericidal drugs, penicillin, ciprofloxacin and kanamycin, stimulate a greater production of ROS in the presence of a functional version of spr1178. Bacteriostatic antibiotics like tetracycline and chloramphenicol failed to induce ROS irrespective of the functional status of spr1178. Even though the transformation of the spr1178 nonsense mutation under a background of mutated PBPs did not reveal a direct role in resistance to penicillin, its early inactivation could have provided increased protection against the accumulation of ROS during the selection of resistance by potentially decreasing the availability of free iron. Moreover, the R6M2 mutant further harbors a nonsense mutation in another putative iron uptake system (spr0934; Table 2), which could potentiate the protective effect conferred by the spr1178 inactivation. The analysis of a panel of five penicillin non-susceptible clinical isolates failed to show similar nonsense mutations in spr1178 (data not shown), but this might in part be explained by the obvious growth defect associated with the acquisition of this mutation (Figure 2). It is salient to point out that the exposure to sublethal concentrations of bactericidal antibiotics was shown to induce a decreased expression of iron uptake systems in Pseudomonas aeruginosa (Mikkelsen et al. 2010) and S. pneumoniae (Rogers et al. 2007), so similar gene expression alterations could also potentially occur in clinical isolates to prevent the accumulation of ROS during the early steps of
resistance selection, instead of more drastic events like nonsense mutation as observed in isolates selected *in vitro*.

Our comparative genomic approach revealed that the selection for penicillin resistance in *S. pneumoniae* frequently involves the acquisition of a nonsense mutation in a putative iron transport system that increases the tolerance to antibiotic-induced accumulation of ROS. This tolerance should lead to an increased survival that putatively allows the selection of more important resistance determinants, such as the sequential accumulation of point mutations in PBPs.

**Conclusions**

This study indicates that, for *in vitro* isolates, mutations in PBPs are not sufficient to achieve high level resistance to penicillin. Our study also reveals that penicillin kills cells by producing ROS, possibly through the Fenton reaction since less ROS are produced in resistant mutants in which a putative iron transporter is mutated. The whole genome sequencing data further revealed other mutations that were acquired by at least one mutant and we propose that some of these, or a combination of mutations, could be associated with penicillin resistance along with mutations in PBPs.

**Materials and methods**

**Bacterial strains and culture conditions**

All strains used in this study are listed in Table 4. Pneumoccoci were grown as previously described (Feng et al. 2009). Clones of the *S. pneumoniae* R6 laboratory strain and the clinical isolate *S. pneumoniae* CCRI-1974 (Feng et al. 2009) were used for the laboratory-induced selection of penicillin resistance. The selection of resistance was performed on Zybalski plates containing concentration gradients of PG as described previously for other drugs (Martineau et al. 2000). For subculturing, colonies were picked in the area of highest antibiotic concentrations and streaked onto agar plates containing either the same concentration of antibiotic or a gradient of increased antibiotic concentrations. The MIC of the resistant cells isolated from the plates with the highest concentrations of antibiotic was determined to confirm the resistance
phenotype. Five selection cycles were required to obtain the highly resistant M1 and M2 mutants for each strain.

**Antibiotic susceptibility**

Antibiotic susceptibilities were determined with E-test strips (AB bioMérieux, Stockholm, Sweden) on Müller-Hinton agar plates supplemented with 5% sheep blood using the manufacturer’s instructions. The MICs were further confirmed by the microdilution method according to the Clinical Laboratory Standards Institute (CLSI) guidelines.

**Whole genome sequencing**

Genomic DNAs were prepared from mid-log phase *S. pneumoniae* cultures using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The genome of the R6M1 mutant was sequenced by using the NimbleGen WGS approach. Briefly, DNA from the R6M1 mutant and its progenitor were differentially labeled with fluorescent markers and were co-hybridized on DNA tiling microarrays. Regions hybridizing differently were sequenced by a second round of sequencing hybridization arrays. The sequencing and analysis were performed by NimbleGen (Albert et al. 2005). The genome of the R6M2 mutant was sequenced using the 454 Life Sciences (Roche, Branford, FL, USA) GS-FLX system. The genome sequencing, assemblies, and comparative analysis were performed at the McGill University Genome Quebec Innovation Center. The sequence of R6M2 is available at NCBI under the accession number PRJNA73471. Mutations, deduced from either array hybridizations or massively parallel sequencing, were confirmed by PCR amplification and Sanger sequencing. A circular map of the genome of R6M1 and R6M2 showing the identified mutations is provided (Additional file 1).

**DNA constructs**

The genetic constructs used in this study are described in Additional file 5. Gene inactivation was done by insertional duplication mutagenesis using the nonreplicative pFF3 and pFF6 plasmids. The pFF3 plasmid is a pGEMT easy (Promega) derivative
into which an Eam1105I restriction site was introduced in the multiple cloning site and the ampicillin resistance marker was replaced by the chloramphenicol resistance marker of pEVP3 (Claverys et al. 1995) (a kind gift of D Morrison, University of Illinois at Chicago). The pFF6 plasmid is a derivative of pFF3 in which the chloramphenicol resistance marker was replaced by the kanamycin marker of pDL289 (Buckley et al. 1995) (a kind gift of D Cvitkovitch, University of Toronto). Fragments of the genes to be inactivated were amplified from genomic DNA of *S. pneumoniae* R6 (Additional file 4) and cloned into the multiple cloning sites of pFF3 or pFF6. The Janus cassette (a kind gift from D Morrison, University of Illinois, Chicago) was also used for gene inactivation and gene replacement study in a streptomycin-resistant background in *S. pneumoniae* as described (Sung et al. 2001). Janus is a 1.3-kb cassette with a kanamycin resistance marker and a counterselectable *rpsL* marker conferring streptomycin sensitivity. To generate a streptomycin-resistant background, *S. pneumoniae* strains were transformed with a *rpsL*<sup>+</sup> PCR fragment that was amplified from chromosomal DNA of the streptomycin-resistant strain CP1296 and the selection was done on agar supplemented with 150 μg/ml of streptomycin.

**Genetic transformation**

The penicillin-resistance phenotype was reconstructed by transforming the penicillin-susceptible *S. pneumoniae* R6 with PCR fragments amplified from the penicillin-resistant R6M1 or R6M2 mutants (Additional file 4). Selection was done on plates containing appropriate concentrations of penicillin. Competent cells were obtained by the dilution of an overnight *S. pneumoniae* culture 1:100 in C+Y medium, pH 6.8 (Tomasz and Hotchkiss 1964). The diluted cultures were grown up to the onset of exponential phase before being concentrated ten times and frozen in C+Y, pH 6.8, 15% glycerol. For transformation, competent cells were thawed on ice, diluted ten times with C+Y medium, pH 7.8, and complemented with 2 μg/ml of competence stimulating peptide 1 (csp-1) before being incubated for 15 minutes at 35°C under a 5% CO<sub>2</sub> atmosphere. DNA was added to a final concentration of 2 μg/ml and the cultures were incubated for 1 hour at 30°C. Finally, the cultures were switched to 35°C under a 5% CO<sub>2</sub> atmosphere for 1 hour before being plated on CAT medium containing the
appropriate concentration of antibiotic. The plates were incubated for 48 hours at 35°C under a 5% CO₂ atmosphere and the resistant colonies were picked for further studies.

**Detection of reactive oxygen species**

The intracellular ROS accumulation was measured using the DCF-DA dye (Invitrogen, Grand Island, NY, USA) whose fluorescence is proportional to the level of ROS (Moreira et al. 2009). In a typical experiment, cells were grown to the onset of exponential phase (OD₆₀₀ 0.12) before penicillin, ciprofloxacin, kanamycin, chloramphenicol or tetracycline were added at a final concentration of 0.1, 4.0, 400, 6.0 and 0.25 μg/ml, respectively. One milliliter aliquots were collected at baseline (prior to the addition of antibiotic) and at 1, 2 and 3 hours following the addition of antibiotic. The aliquots were washed once and resuspended in 500 μl of 1× PBS (pH 7.2) containing 5 μM DCF-DA and incubated at 37°C in the dark for 30 minutes. The labeled cells were washed once and resuspended in 500 μl of 1× PBS. The fluorescence signal of a 200 μl aliquot was analyzed using a Victor fluorometer (Perkin-Elmer, Waltham, MA, USA) at 485 nm excitation and 535 nm emission wavelengths. Results are expressed as relative fluorescence units (RFU) and were normalized according to the number of live cells at each time point. A minimum of three independent experiments have been performed for each antibiotic.

**Abbreviations**

DCF-DA, dichlorofluorescein diacetate; MIC, minimum inhibitory concentration; PBP, penicillin-binding protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PG, penicillin G; ROS, reactive oxygen species; SNP, single-nucleotide polymorphism; WGS, whole genome sequencing; WT, wild-type.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**
FF, DL and MO designed the study. FF performed the experiments, analyzed the data and drafted the manuscript; PL and DL revised the manuscript and provided critical comments. All authors approved the final version of the manuscript.

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Figure legends

Figure 1. Nonsense mutation in spr1178 and reduced accumulation of reactive oxygen species induced by bactericidal antibiotics. Drug-induced reactive oxygen species accumulation in *S. pneumoniae*. The DCF-DA fluorescence signals of the *S. pneumoniae* R6<sup>SmR</sup>, 2x2b1a-M2 (green bars) and R6<sup>SmR</sup>, 2x2b1a, spr1178-M2 (yellow bars) transformants following exposure to (a) 0.1 μg/ml penicillin, (b) 4.0 μg/ml ciprofloxacin, (c) 400.0 μg/ml kanamycin, (d) 6.0 μg/ml chloramphenicol, and (e) 0.25 μg/ml tetracycline were measured prior to antibiotic exposure (time zero) and 1 hour, 2 hours, and 3 hours following addition of the antibiotics. The DCF-DA fluorescence signals of the *S. pneumoniae* R6<sup>SmR</sup>, 2x2b1a-M2 (blue bars) and R6<sup>SmR</sup>, 2x2b1a, spr1178-M2 (orange bars) transformants untreated cultures measured at each time point are indicated as control. Results are the average of at least three independent experiments. RFU, relative fluorescence units.

Figure 2. Nonsense mutation in spr1178 and growth defect in penicillin-resistant *S. pneumoniae*. (a) The growth kinetics of *S. pneumoniae* R6 wild-type, *S. pneumoniae* R6<sup>SmR</sup>, 2x2b1a-M2 and *S. pneumoniae* R6<sup>SmR</sup>, 2x2b1a, spr1178-M2 was followed by measuring the optical density of the cultures every hour for a 20-hour period. (b) The reversion of the spr1178 nonsense mutation to a wild-type sequence decreased the growth defect of the R6M1 mutant. Results are the average of at least three independent measurements.
Table 1. Susceptibility levels of *S. pneumoniae* isolates

<table>
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<tr>
<th>S. pneumoniae strain</th>
<th>PG (μg/ml)</th>
<th>CT (μg/ml)</th>
<th>EM (μg/ml)</th>
<th>CI (μg/ml)</th>
<th>KM (μg/ml)</th>
<th>TC (μg/ml)</th>
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</table>

Results are the average of at least three independent measurements. WT, wild-type; PG, penicillin G; CT, cefotaxime; EM, erythromycin; CI, ciprofloxacin; KM, kanamycin; TC, tetracycline; LZ, linezolid.
Table 2. Mutations identified in R6M1 and R6M2 penicillin-resistant mutants

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<td>Q552E</td>
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<td>spr0509</td>
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<td>C109T</td>
<td>P37S</td>
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<td>spr0598</td>
<td>GTP-binding protein (TypA/BipA) (tyrosine phosphorylated</td>
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<td>protein A)</td>
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<td>spr0666</td>
<td>ABC transporter ATP-binding protein - cell division (FtsE)</td>
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<td>C410T</td>
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<td>C575T</td>
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<td>spr0917</td>
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<td>spr0934</td>
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<td>Position</td>
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<td>Type</td>
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<td>spr1092</td>
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<td>Signal recognition particle (Fifty four homolog)</td>
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<td>C82T</td>
<td>Q28*</td>
<td>C82T</td>
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<td>N-Acetylmuramoylase, lyase subunit, truncation</td>
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<td>spr1240</td>
<td>Alanyl-tRNA synthetase</td>
<td>A139G</td>
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<td>spr1254</td>
<td>ABC transporter ATP-binding protein-phosphate transport (PstB)</td>
<td>G499A</td>
<td>G167S</td>
<td>C614T</td>
<td>T205I</td>
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<td>spr1260</td>
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<td>T597C</td>
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<td>spr1272</td>
<td>N-Acetylglucosamine-6-phosphate isomerase</td>
<td>G385A</td>
<td>G129R</td>
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<td>spr1384</td>
<td>UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysine ligase</td>
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<td>M332I</td>
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<td>spr1423</td>
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<td>A673G</td>
<td>M225V</td>
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<td>spr1453</td>
<td>Major facilitator superfamily transporter</td>
<td>T91C</td>
<td>F31L</td>
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<td>G140E</td>
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<td>spr1517</td>
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<td>C1245A</td>
<td>D415E</td>
<td>C1184T</td>
<td>A395V</td>
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<td>C1528A</td>
<td>G665D</td>
<td>G1303A</td>
<td>G435S</td>
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<td>spr1587</td>
<td>Conserved hypothetical protein</td>
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<td>SYN</td>
<td>A1351G</td>
<td>T451A</td>
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<td>ABC transporter ATP-binding protein - oligopeptide transport</td>
<td>C575A</td>
<td>P192Q</td>
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<td>ABC transporter membrane-spanning permease - oligopeptide transport</td>
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<td>T399deletion</td>
<td>frameshift</td>
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<td>spr1862</td>
<td>Competence protein</td>
<td>A51G</td>
<td>SYN</td>
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<td>spr1886</td>
<td>Degenerate transposase</td>
<td>A314G</td>
<td>*105W</td>
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<tr>
<td>spr1888</td>
<td>DNA mismatch repair protein (HexA)</td>
<td>C2183T</td>
<td>T728I</td>
<td>C976T</td>
<td>Q326*</td>
<td></td>
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<tr>
<td>spr1991</td>
<td>Glycerol kinase</td>
<td>GG78TT</td>
<td>E27*</td>
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</table>

Asterisks and SYN indicate nonsense mutations and synonymous mutations, respectively.
<table>
<thead>
<tr>
<th>Locus name</th>
<th>R6M1</th>
<th>R6M2</th>
<th>1974M1</th>
<th>1974M2</th>
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<tbody>
<tr>
<td>PBP1a</td>
<td>G544R</td>
<td>W411*</td>
<td>E248*</td>
<td>E158*</td>
</tr>
<tr>
<td>PBP2x</td>
<td><strong>R384G, V518I, Q552E</strong></td>
<td>Q281P, A369V, <strong>R384G, R426C, V518I</strong></td>
<td><strong>F388L, Q552E, V573L, V587L, G601V</strong></td>
<td><strong>A507V, P535L, Q552E</strong></td>
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<tr>
<td>PBP2b</td>
<td>D415E, G665D</td>
<td>A395V, G435S, <strong>T451A</strong></td>
<td><strong>G435S, T451A</strong></td>
<td><strong>T431D, T451A, L492F, Q633E</strong></td>
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<tr>
<td>Spr1178</td>
<td><strong>Q28</strong></td>
<td><strong>Q28</strong></td>
<td><strong>Q28</strong></td>
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<td>Spr1254</td>
<td>G167S</td>
<td>T205I</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>Spr1888</td>
<td>T728I</td>
<td>Q326*</td>
<td>WT</td>
<td>WT</td>
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</table>

*Mutations are shown as amino acid changes with their corresponding position in the protein. Asterisks indicate nonsense mutations. Mutations that are common in at least two strains are in bold.*
Table 4. Minimal inhibitory concentrations to penicillin of *Streptococcus pneumoniae* mutants and transformants

<table>
<thead>
<tr>
<th>Strain or transformant</th>
<th>Description</th>
<th>MIC to PG (μg/ml)</th>
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<tbody>
<tr>
<td>R6</td>
<td>Wild-type</td>
<td>0.023</td>
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<tr>
<td>CCR11974</td>
<td><em>S. pneumoniae</em>, sensitive clinical isolate</td>
<td>0.023</td>
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<td>R6M1</td>
<td>R6 clone selected <em>in vitro</em> for PG resistance</td>
<td>2.0</td>
</tr>
<tr>
<td>R6M2</td>
<td>R6 clone selected <em>in vitro</em> for PG resistance</td>
<td>2.0</td>
</tr>
<tr>
<td>1974M1</td>
<td>1974 clone selected <em>in vitro</em> for PG resistance</td>
<td>2.0</td>
</tr>
<tr>
<td>1974M2</td>
<td>1974 clone selected <em>in vitro</em> for PG resistance</td>
<td>2.0</td>
</tr>
<tr>
<td>CP1250</td>
<td><em>S. pneumoniae</em> that contains a spontaneous mutation in the rpsL gene that confers resistance to SM</td>
<td></td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x-M1&lt;/sup&gt;</td>
<td>R6-WT transformed with <em>pbp2x</em> PCR fragments from R6M1 (contains all three missense mutations present in PBP2x of R6M1)</td>
<td>0.06</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x-M2&lt;/sup&gt;</td>
<td>R6-WT transformed with <em>pbp2x</em> PCR fragments from R6M2 (contains just three mutations - Q281P, A369V, R384G - out of five mutations present in PBP2x of R6M2)</td>
<td>0.06</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x2b-M1&lt;/sup&gt;</td>
<td>R6&lt;sup&gt;2x-M1&lt;/sup&gt; transformed with <em>pbp2b</em> PCR fragments from R6M1 so all missense mutations present in PBP2b of R6M1 transformed into this transformant</td>
<td>0.125</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x2b-M2&lt;/sup&gt;</td>
<td>R6&lt;sup&gt;2x-M2&lt;/sup&gt; transformed with <em>pbp2b</em> PCR fragments from R6M2 so all missense mutations present in PBP2b of R6M2 transformed into this transformant</td>
<td>0.125</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x2b-M2&lt;/sup&gt;, 1a::Janus</td>
<td>R6&lt;sup&gt;2x2b-M2&lt;/sup&gt; knocked out in <em>pbp1a</em> by Janus cassette, KM&lt;sup&gt;R&lt;/sup&gt;SM&lt;sup&gt;R&lt;/sup&gt;</td>
<td>0.125</td>
</tr>
<tr>
<td>R6&lt;sup&gt;SmR, 2x2b1a-M2&lt;/sup&gt;, 1a::Janus</td>
<td>R6&lt;sup&gt;2x2b-M2&lt;/sup&gt; subjected to second step transformation with <em>pbp1a</em> of the Janus cassette with <em>pbp1a</em> of R6M2, KM&lt;sup&gt;R&lt;/sup&gt;SM&lt;sup&gt;R&lt;/sup&gt;</td>
<td>0.125</td>
</tr>
<tr>
<td>R6&lt;sup&gt;SmR, 2x2b1a,spr1178-M2&lt;/sup&gt;, 1a::Janus</td>
<td>R6&lt;sup&gt;2x2b-M2&lt;/sup&gt; transformed with spr1178 PCR fragments from R6M2 (contains a Q28* nonsense mutation in spr1178), SM&lt;sup&gt;R&lt;/sup&gt;</td>
<td>0.125</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x1b, spr1254-M2&lt;/sup&gt;</td>
<td>R6&lt;sup&gt;2x2b-M2&lt;/sup&gt; transformed with pstB PCR fragment from R6M2 (contains a T205I mutation in PstB)</td>
<td>0.125</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x1b, spr1254, spr1178-M2&lt;/sup&gt;</td>
<td>R6&lt;sup&gt;2x2b-M2&lt;/sup&gt; transformed with spr1178 and pstB PCR fragments from R6M2 (contains the substitutions Q28* in spr1178 and T205I in PstB)</td>
<td>0.125</td>
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<tr>
<td>R6M1&lt;sup&gt;SmR, 1a-WT&lt;/sup&gt;</td>
<td>R6M1 co-transformed with a <em>pbp1a</em> PCR fragment from R6-WT and a <em>rpsL</em> streptomycin resistance marker, SM&lt;sup&gt;R&lt;/sup&gt;</td>
<td>1.0</td>
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<tr>
<td>R6&lt;sup&gt;ko in spr1178&lt;/sup&gt;</td>
<td>R6-WT knocked out in spr1178 , CM&lt;sup&gt;R&lt;/sup&gt;</td>
<td>0.023</td>
</tr>
<tr>
<td>R6&lt;sup&gt;ko in spr1254&lt;/sup&gt;</td>
<td>R6-WT knocked out in spr1254 , KM&lt;sup&gt;R&lt;/sup&gt;</td>
<td>0.023</td>
</tr>
<tr>
<td>R6&lt;sup&gt;ko in spr1888::Janus&lt;/sup&gt;</td>
<td>R6-WT knocked out in spr1888 by Janus cassette, KM&lt;sup&gt;R&lt;/sup&gt;SM&lt;sup&gt;R&lt;/sup&gt;</td>
<td>0.023</td>
</tr>
</tbody>
</table>

*Results are the average of at least three independent measurements. Asterisks indicate nonsense mutations. PG, penicillin; KM, kanamycin; SM, streptomycin.*
Figure 1
Figure 2
Additional files

Additional file 1: Circular maps of the genome of R6M1 and R6M2.

Additional file 2: Chronological appearance of PBP mutations according to the levels of penicillin resistance in R6M2.

Additional file 3: PBP1a-targeting Janus cassette.

Additional file 4: Oligonucleotides used in this study.

Additional file 5: Plasmids used in this study.
Additional file 1. Circular maps of the genome of R6M1 and R6M2.
Circular maps (CG view) of the R6M1 (A) and R6M2 (B) genomes in which the genes containing non-synonymous mutations are shown. The genes located on the forward strand are indicated in red and those located on the reverse strand are shown in blue. Recurrent mutations in both mutants are shown in bold. polA, DNA polymerase A; IS1167, transposase; pspA, surface protein precursor; mutL, DNA mismatch repair protein; xylS, alpha xylosidase; pbp, penicillin binding protein; pheS, phenylalanyl-tRNA synthetase; typA, tyrosin phosphorylated protein A; ftsE, cell division ATP binding protein; rpsA, 30S ribosomal protein S1; dacA, D-alanyl-D-alanine carboxypeptidase; pyrD, dihydroorotate dehydrogenase; rnr, exoribonuclease R; ccl, citrulline cluster-linked gene; ABC-SBP, ATP binding cassette-substrate binding protein; rbgA, ribosomal biogenesis GTPase; rnc, ribonuclease III; licD2, phosphorylcholine transferase; alaS, alanyl-tRNA synthetase; pstB, phosphate transporter ATP-binding protein; nagB, N-acetylg glucosamine 6-phosphate isomerase; murE, UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysine ligase; ami, N-acetylmuramoyl-L-alanine amidase; cglC, competence protein; hexA, hexoaminidase; glpK, glycerol kinase. The maps have been generated with the CG view software (http://www.simgene.com/CGView).
### Additional file 2. Chronological appearance of PBPs mutations according to the levels of penicillin resistance in R6M2.

<table>
<thead>
<tr>
<th>Interval stage of M2</th>
<th>PBP</th>
<th>PBP2x</th>
<th>PBP2b</th>
<th>PBP1a</th>
<th>Spr1178</th>
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</thead>
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<tr>
<td>Level 1</td>
<td>MIC;0.06</td>
<td>A842C</td>
<td>Q281P</td>
<td>NO</td>
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<tr>
<td>Level 2</td>
<td>MIC;0.125</td>
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<td>Q281P</td>
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<td>Level 3</td>
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<td>C1106T</td>
<td>A369V</td>
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<tr>
<td>Level 5</td>
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<td>A369V</td>
<td>R384G</td>
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<tr>
<td>Level 6</td>
<td>MIC;2.0</td>
<td>A842C</td>
<td>Q281P</td>
<td>A369V</td>
<td>R384G</td>
</tr>
</tbody>
</table>

Mutations are shown as nucleotide changes in the first line and the corresponding amino acid changes shown in italic on the second line. Asterix (*) indicates nonsense mutations. NO indicates no mutations. MICs are in µg/ml.
Additional file 3. PBP1a-targeting Janus cassette.

The *rpsL* and *kan*, modules of the Janus cassette are shown. Primers used to amplify two targeting fragments are indicated at the termini of those PCR products. Top, PCR fragment used to construct R6<sup>2x2b-M2, 1a::Janus</sup> transformant. Middle, *pbp1a* chromosomal region. Bottom, a 8,160-bp fragment containing *pbp1a* flanked with 3Kb upstream (UPS) and downstream (DNS) amplified fragments from chromosomal DNA of R6M2 with primers FF288 and FF289, This 8160 bp fragment was used for reintroduction of *pbp1a* of R6M2 to replace the Janus Cassette.
### Additional file 4. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Targeted Locus</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF152</td>
<td>pbp2b-F</td>
<td>CTAATTCAATGGATGGATTT</td>
</tr>
<tr>
<td>FF153</td>
<td>pbp2b-R</td>
<td>ATGAGACTGATTTGTATCGA</td>
</tr>
<tr>
<td>FF154</td>
<td>pbp2x-F</td>
<td>ATGAAGTGCAAAAGAGTA</td>
</tr>
<tr>
<td>FF155</td>
<td>pbp2x-R</td>
<td>TTAGTCTCCTAAAGTATGT</td>
</tr>
<tr>
<td>FF156</td>
<td>pbp1a-F</td>
<td>TTATGTGGTGCTGTGTTGAG</td>
</tr>
<tr>
<td>FF157</td>
<td>pbp1a-R</td>
<td>ATGAACAAAAACAGATTCTG</td>
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For pbp sequencing and PCR transformation

<table>
<thead>
<tr>
<th>Primer</th>
<th>Targeted Locus</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF284</td>
<td>1KbUP- pbp1a -F</td>
<td>GTAGCAGATGACCTTGGCAATCAGTCTTACAGCT</td>
</tr>
<tr>
<td>FF285</td>
<td>UP- pbp1a -JANUS-KM-R</td>
<td>ATTTCTCCTGGAATAGGCATAGCACATTTACATCCAGATTTTT</td>
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<tr>
<td>FF286</td>
<td>DN- pbp1a -JANUS-SM-F</td>
<td>AAAAGCATAAGGAAAGGGGCCCTAGGTTGTTTTACCAACTAATAA</td>
</tr>
<tr>
<td>FF287</td>
<td>1Kb- pbp1a -R</td>
<td>CCCCCTGTGTTCAATCAGCGAGGAATTCAAGCA</td>
</tr>
<tr>
<td>FF212</td>
<td>JANUS-F</td>
<td>TCTATGCCTATTCAGAGAAATTGAT</td>
</tr>
<tr>
<td>FF213</td>
<td>JANUS-R</td>
<td>CTAGGGCCCCCTTCTCTATGCTTTTGAC</td>
</tr>
<tr>
<td>FF288</td>
<td>3KbUP- pbp1a -F</td>
<td>TCAACAGTCGACCACTACCCGC</td>
</tr>
<tr>
<td>FF289</td>
<td>3KbDN- pbp1a -R</td>
<td>ATAGGCGGATTGAAATGATTACCTC</td>
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For gene replacement using Janus cassette

<table>
<thead>
<tr>
<th>Primer</th>
<th>Targeted Locus</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF235</td>
<td>2.5KbUP-spr1178-F</td>
<td>AAAGTTGTAAACCTCCGCAAACACC</td>
</tr>
<tr>
<td>FF236</td>
<td>2.5KbDN-spr1178-R</td>
<td>AACACCGATATCGACGAGGTA</td>
</tr>
<tr>
<td>FF237</td>
<td>2.5KbUP-spr1254-F</td>
<td>TTATCATAATGGCAACGTGTC</td>
</tr>
<tr>
<td>FF238</td>
<td>2.5KbDN-spr1254-R</td>
<td>GTCAATGTCCAAGGGAGG</td>
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</table>

For point mutation transformation of Non-PBPs

<table>
<thead>
<tr>
<th>Primer</th>
<th>Targeted Locus</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF158</td>
<td>Spr1254-F-KO</td>
<td>CTCAACACATGGTCTTCTCTAG</td>
</tr>
<tr>
<td>FF159</td>
<td>Spr1254-R-KO</td>
<td>GTCAACCGTCCGAAATCAAC</td>
</tr>
<tr>
<td>FF207</td>
<td>Spr1178-F-KO</td>
<td>GTTTTCAACACGTCCATAAT</td>
</tr>
<tr>
<td>FF208</td>
<td>Spr1178-R-KO</td>
<td>CTAAGTTTATGCTTTTTCATTTT</td>
</tr>
</tbody>
</table>

Underlined sequences correspond to complement of Kanamycin (KM) and Streptomycin (SM) parts of the Janus cassette. UP, upstream; DN, downstream; F, forward; R, reverse.
### Additional file 5. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid or cassette</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Janus Cassette</td>
<td>A 1.3-kb cassette containing a KM resistance marker and conferring SM sensitivity for gene replacement through negative selection in <em>S. pneumoniae</em>.</td>
<td>(Sung et al. 2001)</td>
</tr>
<tr>
<td>pFF3</td>
<td><em>S. pneumoniae</em> non-replicative vector that contains a CM resistance marker</td>
<td>Generated in our lab (unpublished data)</td>
</tr>
<tr>
<td>pFF6</td>
<td>pFF3 in which the CM resistance marker was replaced with a KM resistance marker.</td>
<td>Generated in our lab (unpublished data)</td>
</tr>
<tr>
<td>pFF3-1178 KO</td>
<td>pFF3 carrying an internal segment of spr1178</td>
<td>This study</td>
</tr>
<tr>
<td>pFF6-1254 KO</td>
<td>pFF6 carrying an internal segment of spr1254</td>
<td>This study</td>
</tr>
</tbody>
</table>

KM, Kanamycin; SM, Streptomycin; CM, Chloramphenicol.
Chapter VIII. Genome analysis and reconstruction of cefotaxime resistant in *Streptococcus pneumoniae*

This chapter contains a manuscript that has been published in “Journal of Antimicrobial Chemotherapy” (2013) as a research article.

8.1 Résumé

Les génomes de deux souches mutantes de *Streptococcus pneumoniae* sélectionnées pour la résistance au céfotaxime en conditions de laboratoire et de deux transformants qui ont été transformés avec l’ADN génomique de mutants résistants au céfotaxime ont été déterminés par séquençage du génome. L’analyse des assemblages des génomes a montré des mutations dans les gènes codant pour les protéines liant à la pénicilline (PLP), 2x, 2a et 3, dont *pbp2X* était le seul gène muté dans tous les mutants. La transformation des allèles modifiés des gènes PLPs dans *S. pneumoniae* R6 a confirmé le rôle des mutations PLP dans la résistance au céfotaxime mais ceux-ci n’étaient pas suffisants pour donner le niveau de résistance retrouvé chez le mutant initial. Trente-et-un gènes supplémentaires étaient mutés dans au moins un des quatre génomes séquencés. Les déterminants de la résistance indépendante des PLPs semblaient être spécifiques pour chaque lignée. Les mutations dans les gènes spr1333, spr0981, spr1704 et spr1098 qui codent respectivement pour un peptidoglycan GlcNAc déacetylase, une glycosyltransférase, un transporteur ABC et une sortase ont été observées dans les mutants résistants. Des expériences de transformation ont démontré l’implication de ces gènes dans la résistance au céfotaxime et a permis la reconstruction du plein niveau de la résistance observé dans les souches parentales résistantes. Ce travail d’analyse du génome entier couplé à des études fonctionnelles a permis à découvrir de nouveaux gènes de résistance au céfotaxime chez *S. pneumoniae*.
8.2 Article
Genomic analysis and reconstruction of cefotaxime resistance in *Streptococcus pneumoniae*

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Abstract

Objectives To identify non-penicillin binding protein mutations contributing to resistance to the third generation cephalosporin cefotaxime in Streptococcus pneumoniae at the genome wide scale.

Methods The genomes of two in vitro Streptococcus pneumoniae cefotaxime resistant isolates and of two transformants serially transformed with the gDNA of cefotaxime-resistant mutants were determined by next generation sequencing. A role in cefotaxime resistance for the mutations identified was confirmed by reconstructing resistance in a cefotaxime susceptible background.

Results Analysis of the genome assemblies revealed mutations in genes coding for the penicillin-binding proteins (PBPs) 2x, 2a and 3, of which \textit{pbp2x} was the only mutated gene common to all mutants. The transformation of altered PBP alleles into \textit{S. pneumoniae} R6 confirmed the role of PBP mutations in cefotaxime resistance but these were not sufficient to fully explain the levels of resistance. Thirty-one additional genes were found to be mutated in at least one of the four sequenced genomes. Non-PBP resistance determinants appeared to be mostly lineage-specific. Mutations in \textit{spr1333}, \textit{spr0981}, \textit{spr1704} and \textit{spr1098} coding respectively for a peptidoglycan GlcNAc deacetylase, a glycosyltransferase, an ABC transporter, and a sortase were implicated in resistance by transformation experiments and allowed the reconstruction of full level of resistance as observed in the parent resistant strains.

Conclusions This whole genome analysis coupled to functional studies has allowed the discovery of both known and novel cefotaxime resistance genes in \textit{S. pneumoniae}. 

Keywords: *Streptococcus pneumoniae*, Next generation sequencing, Resistance, Cefotaxime, Peptidoglycan deacetylase, Sortase, Glycosyltransferase, ABC transporter.
Introduction

*Streptococcus pneumoniae* is a leading cause of bacteremia, meningitis, upper respiratory tract infections and otitis media worldwide and is responsible for around 11% of all deaths in children of less than five years old. (O'Brien et al. 2009) Antimicrobial therapy based on β-lactam antibiotics is the recommended treatment regimen against pneumococcal pneumonia, the most active agents being the penicillin amoxicillin and the third generation cephalosporins cefotaxime and ceftriaxone. (Lynch and Zhanel 2009) Unfortunately, the control of pneumococcal infections is complicated by the dissemination of antibiotic resistance that have occurred within the past three decades. In comparison to the marked escalation in penicillin non-susceptible pneumococci, the rates of resistance to cefotaxime remain globally low. Some regions or countries are nonetheless experimenting noted rises in cefotaxime resistance which are mainly due to the spread of few successful resistant clones. (Lynch and Zhanel 2009) A precise understanding of the mechanisms leading to cefotaxime resistance is thus warranted.

β-lactams interact with penicillin-binding proteins (PBPs), a group of membrane-associated cytoplasmic proteins central to the synthesis of the bacterial cell wall and whose inhibition results in growth arrest and lysis. The primary resistance determinant to β-lactams in *S. pneumoniae* involves the production of mosaic PBPs with reduced antibiotic binding affinities that are acquired by lateral gene transfer events from closely related streptococcal species. (Chambers 1999; Hakenbeck et al. 1999) Six PBPs have been identified in *S. pneumoniae* (PBPs 1a, 1b, 2a, 2b, 2x, 3), of which alterations in PBPs 2x, 2b and 1a account for virtually all β-lactam resistance. While PBP2b is the primary target of penicillin, cephalosporins are mainly interacting with PBP2x. (Hakenbeck et al. 1987; Smith and Klugman 2005; Stanhope et al. 2008) Mutations in PBP1a is required for higher levels of resistance for both penicillin and cefotaxime. (Smith and Klugman 1998) Other reports have described a role for low affinity PBP3 in resistance to cefotaxime, (Krauss and Hakenbeck 1997) and for low affinity PBP2a in resistance to both penicillin and cefotaxime. (Laible and Hakenbeck 1987; Laible et al. 1991; Krauss and Hakenbeck 1997; Hakenbeck et al. 1998; Chesnel
et al. 2005; Smith et al. 2005b) Nonetheless, the level of resistance conferred by mosaic PBPs cannot always account for the high levels of resistance observed in some resistant isolates and other non-PBP contributors to resistance have been reported. The cell wall of penicillin non-susceptible isolates is often highly enriched in branched chain muropeptides, a phenomenon that has been linked to mosaic alleles of the murM gene. (Filipe and Tomasz 2000; Smith and Klugman 2001) Furthermore, mutations in a peptidoglycan N-acetylglucosamine (GlcNAc) deacetylasel, (Tait-Kamradt et al. 2009) a peptidoglycan O-acetyltransferase,(Crisostomo et al. 2006) a putative glycosyltransferase,(Grebe et al. 1997) a serine threonine kinase,(Dias et al. 2009) a histidine protein kinase part of a two-component signal transducing system,(Guenzi et al. 1994) a phosphate ABC transporter,(Soualhine et al. 2005) or in a putative iron permease,(Fani et al. 2011) have also been shown to alter the level of susceptibility to β-lactams or to be implicated in the development of resistance.

This manifold of resistance determinants against β-lactams is rendering sequencing of sensitive and resistant strains an attractive approach to provide a complete view about the cellular events leading to resistance. (Albert et al. 2005; Mwangi et al. 2007; Feng et al. 2009; Billal et al. 2011; Fani et al. 2011; Sauerbier et al. 2012) In this study, we used next generation sequencing (NGS) with resistance reconstruction by whole genome transformation to narrow down the list of mutations involved in resistance to the third generation cephalosporin cefotaxime. We report the identification of known resistance determinants as well as new non-PBP mutations implicated in resistance.
Materials and Methods

Bacterial strains, culture conditions and MIC determination

Unless otherwise stated, pneumococci were grown in brain heart infusion broth (BHI, Difco), or on blood agar plates as described previously (Munoz et al. 1992). Cultures were incubated for 16 to 24 h in a 5% CO₂ atmosphere at 35°C. The R6M1, R6M2 and R6M3 cefotaxime-resistant mutants have been generated from \textit{S. pneumoniae} R6 by stepwise increments on Zybalski plates supplemented with concentration gradients of cefotaxime as described previously. (Martineau et al. 2000; Fani et al.) For subculturing, colonies were picked in the area of highest antibiotic concentrations and streaked onto blood agar containing either the same concentration of cefotaxime or a gradient of increasing cefotaxime concentrations. The minimum inhibitory concentration (MIC) of the resistant cells isolated from the plates with the highest concentrations of antibiotic was determined to confirm the phenotype of resistance. Five selection cycles were required to obtain the highly resistant R6M1, R6M2 and R6M3 mutants. The cefotaxime MIC was determined with Etest strips (AB Biodisk) on Müller-Hinton agar plates supplemented with 5% sheep blood using manufacturer’s instructions. The MICs were further confirmed by the microdilution method according to the Clinical Laboratory Standards Institute (CLSI) guidelines.

DNA Transformation

To induce competence, bacteria were cultured at 35°C in C+Y medium at pH 6.8, (Tomasz and Hotchkiss 1964) until the onset of exponential phase. The cells were concentrated tenfold and resuspended in C+Y medium at pH 7.8 supplemented with 15% glycerol, aliquoted and frozen at -80°C. For transformation, competent cells were thawed on ice, diluted ten times with C+Y medium pH 7.8 and stimulated with 2mg/L of competence stimulating peptide 1 (csp-1) for 15 min at 35°C under a 5% CO₂ atmosphere. Genomic DNA or PCR product was added to a final concentration of 2 mg/L and the cultures were incubated for 1 hour at 30°C, followed by 1 h at 35°C under a 5% CO₂ atmosphere. The cultures were then plated on casa-amino acids tryptone (CAT) agar with the appropriate concentration of cefotaxime and incubated
for 48 h at 35°C under a 5% CO₂ atmosphere. High molecular weight genomic DNA from the R6M1, R6M2 and R6M3 cefotaxime-resistant mutants was extracted with the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions and used to serially transform *S. pneumoniae* R6 to yield the TM1 and TM2 series of cefotaxime-resistant transformants along with the T1M3 transformant.

For the targeted transformation of PCR products conferring phenotypes less amenable to selection, we employed a co-transformation strategy whereby the PCR products of interest were co-transformed with a PCR product covering the *rpsL*+ allele (coding for ribosomal protein S12) of *S. pneumoniae* CP1296.(Sung et al. 2001) The *rpsL*+ allele of *S. pneumoniae* CP1296 codes for a Lys57Thr mutation conferring resistance to streptomycin that enables selecting for transformants under streptomycin pressure. The transformation efficiency of *S. pneumoniae* R6 is such that as much as 25% of the streptomycin-resistant clones also integrated the PCR product of interest as confirmed by Sanger sequencing. Only clones having integrated an *rpsL*+ allele along with the PCR product of interest were conserved.

**Whole Genome Sequencing**

Genomic DNAs were prepared from mid-log phase *S. pneumoniae* cultures using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer’s instructions. The genomes of R6M1, T3M1 and T3M2 were sequenced using the 454 Life Sciences (Roche) GS-FLX Titanium system while the genome of R6M3 was sequenced with a HiSeq1000 next-generation sequencer (Illumina) using a 101-nucleotides paired-end reads protocol. The sequencing, assemblies, and comparative analysis for the R6M1, T3M1 and T3M2 strains were performed at the Genome Quebec Innovation Center from McGill University. The sequencing and bioinformatic work for the R6M3 mutant was performed at the Centre de Recherche en Infectiologie of Université Laval. Sequence reads from each strain were aligned to the reference genome *S. pneumoniae* R6,(Hoskins et al. 2001) using the software bwa (bwa aln, version 0.5.9) with default parameters.(Li and Durbin 2009) The maximum number of mismatches was 4, the seed length was 32 and 2 mismatches were allowed within the
seed. The detection of single nucleotide polymorphisms was performed using samtools (version 0.1.18), bcftools (distributed with samtools) and vcfutils.pl (distributed with samtools), (Li et al. 2009) with a minimum of three reads to call a potential variation prior to further analysis. The sequence data are available at the EMBL European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the study accession number ERP001564; samples ERS153540, ERS153541, ERS153542 and ERS153543 for S. pneumonias T3M1, R6M1, T3M2 and R6M3 respectively. Mutations deduced from massively parallel sequencing were confirmed by PCR amplification and Sanger sequencing.
Results

Selection and whole-genome sequencing of *S. pneumoniae* resistant to cefotaxime

The *S. pneumoniae* R6M1, R6M2 and R6M3 cefotaxime-resistant mutants were selected by exposure of *S. pneumoniae* R6 wild-type (WT) to stepwise increments of cefotaxime until reaching a final cefotaxime MIC of 4mg/L (R6M1 and R6M2) or 8mg/L (R6M3) (Table 1). It has not been possible to obtain mutants resistant to higher levels of cefotaxime. The three cefotaxime-resistant strains were 3-10 fold cross-resistant to penicillin (Table 1) but remained susceptible to several other non β-lactam antibiotics (data not shown).

The reconstruction of antibiotic resistance by transformation of susceptible bacteria with high molecular weight DNA derived from resistant strains facilitates the discrimination of mutations involved in resistance from bystander mutations selected during the acquisition of resistance (Billal et al. 2011). We transformed genomic DNA (gDNA) derived from R6M1, R6M2 and R6M3 into *S. pneumoniae* R6 WT recipients, selected transformants under cefotaxime pressure and sequenced the genome of the transformants displaying the same level of resistance as the parent mutant by NGS (see Materials and Methods). A total of three rounds of transformation were required to fully reconstruct the high level cefotaxime resistance of the R6M1 and R6M2 mutants, leading to the T3M1 and T3M2 transformants, respectively (Table 1 and Fig. S1). However we failed to reconstruct resistance found in R6M3 by whole genome transformation and could never obtained transformants displaying a cefotaxime MIC higher than 0.25mg/L (Table 1 and Fig. S1).

We sequenced R6M1, its transformant T3M1 resistant to similar levels to cefotaxime (Table 1), T3M2 and R6M3 (Table 2). The genome sequence of R6M1 revealed eleven non-synonymous mutations of which six were transferred to the T3M1 transformant (Table 2). These included four missense mutations in *pbp2x*, a missense mutation in *pbp2a* and a non-sense mutation in *spr1098*. The T3M1 strain also acquired spontaneous mutations in *spr1120* and *spr1870* during its selection under cefotaxime pressure. The genome sequence of T3M2 revealed a total of eleven non-synonymous
mutations transferred from R6M2 (Table 2), including mutations in three PBP genes (*pbp2x*, *pbp2a* and *pbp3*). Of the eleven mutations, two (spr0652 and spr0839) occurred spontaneously during the selection of T3M2 since they were absent in R6M2 as determined by targeted sequencing of these genes in the parent mutant.

Since we could not obtain transformants derived from R6M3, we sequenced the genome of this mutant and found a total of seventeen non-synonymous mutations compared to its *S. pneumoniae* WT parent (Table 2). In contrast to R6M1 and R6M2, *pbp2x* was the only PBP gene mutated in R6M3 (Table 2). In fact, *pbp2x* was the only common mutated gene in every strain sequenced with the M289T and Q552E substitutions being shared in the sequenced strains (Table 2). The only other genes mutated in more than one strain were the *pbp2a* gene in T3M1 and T3M2, and the spr0180 and spr1333 genes mutated in T3M2 and R6M3 (Table 2). Every mutation identified in R6M1, T3M1, T3M2 and R6M3 was confirmed by PCR amplification and conventional DNA sequencing.

**Penicillin-binding proteins and cefotaxime resistance**

The targeted sequencing of the mutations derived from R6M1 at each round of transformation revealed that the Q552E substitution in PBP2x and the A463E change in PBP2a appeared first in the T1M1 transformant selected at 0.5mg/L cefotaxime (Table 3). For the transformants of the R6M2 mutant, the only mutation transferred during the first round of transformation selected with 0.125mg/L was the T550A substitution in PBP2x (Table 4). The second round transformants had MIC of 1.0mg/L (T2M1) or 2.0mg/L (T2M2) and the entire set of mutations in PBP2x was transferred (Tables 3 and 4). The second level transformant T2M2 also acquired missense mutations in its *pbp2a* and *pbp3* genes (Table 4). Transformation experiments of *S. pneumoniae* R6 WT with *pbp* genes amplified from the R6M1, R6M2 or R6M3 mutants were conducted to assess the contribution of each PBP mutations to cefotaxime resistance. The *pbp2x* gene was amplified from genomic DNAs derived from the R6M1, R6M2 and R6M3 mutants and the PCR fragments were sequenced to ascertain the presence of the mutations described in Table 2 and transformed into WT
cell. The independent selection of transformants with 0.03mg/L cefotaxime enabled recovering the transformants R6$^{2x-M1}$, R6$^{2x-M2}$ and R6$^{2x-M3}$ all with an increased MIC at 0.5mg/L (Table 5). In a second round of transformation, the $pbp2a$ gene from R6M1 was transformed into the recipient R6$^{2x-M1}$. The selection with 0.5mg/L cefotaxime yielded second-level transformants that acquired the PBP2a mutation of R6M1 (A463E) and these R6$^{2x2a-M1}$ transformants had an MIC of 1.0mg/L (Table 5). Similarly, the transformation of the recipient R6$^{2x-M2}$ with a $pbp2a$ PCR fragment amplified from R6M2 yielded the second-level R6$^{2x2a-M2}$ with an MIC of 1.0mg/L (Table 5). A third transformation was performed whereby the PBP3 T242K mutation from R6M2 was introduced into R6$^{2x2a-M2}$ recipient cells. This transformant R6$^{2x2a,pbp3-M2}$ had a cefotaxime MIC of 2.0mg/L, only two folds less than the parent original mutant or transformant (Table 5).

**Non-PBP mutations and cefotaxime resistance**

Mutations in PBPs could only partly explain the levels of cefotaxime resistance observed in the T3M1 and T3M2 transformants or in the R6M3 mutant. Other mutations were thus likely to be involved and we first concentrated on recurrent mutations. The spr1333 gene codes for the peptidoglycan GlcNAc deacetylase PdgA with a nonsense mutation in T3M2 and a frameshift mutation in R6M3 (Table 2). These mutations were selected late in both strains, i.e. after the mutations in PBPs have appeared when the MIC to cefotaxime was higher than 2.0mg/L (Tables 4 and 6). PCR fragments of $pdgA$ amplified from T3M2 were used as a donor DNA for the transformation of R6$^{2x2a,pbp3-M2}$. The selection of transformants at 2.0mg/L cefotaxime enabled recovering the R6$^{2x2a,pbp3,spr1333-M2}$ strain which had a cefotaxime MIC of 4.0mg/L (Table 5), thus allowing a full reconstruction of the cefotaxime resistance of the R6M2 mutant. The only mutation differentiating cells resistant to 1.0mg/L and 2.0mg/L (1M3 and 2M3) while selecting for R6M3 is the deletion in spr1333 (Table 6). Transformation of the spr1333 mutation of R6M3 into 1M3, yielded 1M3$^{spr1333-M3}$ with an increased MIC for cefotaxime (Table 5). Similarly, the introduction of a wild type allele of spr1333 into 2M3 reduced resistance to cefotaxime (Table 5).
Apart from the mutations in \(pbp2x\) and \(pbp2a\), the nonsense mutation in the \(spr1098\) gene coding for sortase A (StrA) is the only other mutation that had been transferred from the R6M1 mutant to its T3M1 transformant which had the same MIC (Tables 1 and 2). We failed to introduce the \(spr1098\) mutation into the \(S.\ pneumoniae\) R6\(^2\)x2a-M1 genetic background. However, transformation of the \(spr1098\) mutation from R6M1 into \(S.\ pneumoniae\) R6 WT increased cefotaxime resistance from 0.023mg/L to 0.25mg/L (Table 5, R6\(^{spr1098\,-M1}\)), while reintroducing a non-mutated version of \(spr1098\) into T3M1 led to a four-fold sensitization to cefotaxime in the transformants (Table 5), indicating \(spr1098\) is involved in resistance.

The mutations within the \(pbp2x\) and \(spr1333\) genes of R6M3 cannot entirely explain the high level of resistance of this mutant. The genome sequence of R6M3 revealed 12 additional non-synonymous mutations that were acquired during the five distinct cefotaxime increments required for selecting R6M3 and we established the order of appearance of these mutations during the last three increments (1M3 at 1.0mg/L, 2M3 at 2.0mg/L and R6M3 at 8.0mg/L) by targeted sequencing (Table 6). The increase in MIC from 1.0mg/L in 1M3 to 2.0mg/L in 2M3 is due to the mutation in \(spr1333\) and we then concentrated on the five mutations (\(spr0180\), \(spr0303\), \(spr0981\), \(spr1358\) and \(spr1754\)) absent from 2M3 but acquired in R6M3 (Table 6). The mutations within the glycosyltransferase \(spr0981\) (CpoA) and the LytA autolysin \(spr1754\) were the most attractive due to prior reports about their role in resistance to \(\beta\)-lactams. (Liu and Tomasz 1987; Jabes and Tomasz 1989; Zahner et al. 1996; Grebe et al. 1997) The introduction of the \(spr0981\) mutation from R6M3 into 2M3 mutant produced the 2M3\(^{spr0981\,-M3}\) transformants which increased cefotaxime MIC to the level found in R6M3 (8.0mg/L) (Table 5). Similarly, introducing a wild type version of \(spr0981\) into R6M3 led to cells being four-fold more sensitive to cefotaxime (Table 5). In contrast, the transformation of the 2M3 mutant with \(spr1754\) PCR fragment amplified from R6M3 had no impact on the level of resistance of 2M3 against cefotaxime (data not shown).
The early selection steps of R6M3 implicated the acquisition of mutations in the membrane spanning domain of the spr1255 ABC transporter (phosphate transport) and in the ATP-binding domain of the spr1704 ABC transporter (oligopeptides transport) (Table 6). Despite several attempts, we were unsuccessful at obtaining clones when transforming R6\textsuperscript{2x}\textsuperscript{-M3} with spr1255 PCR fragments amplified from R6M3. However, the transformation of R6\textsuperscript{2x}\textsuperscript{-M3} with spr1704 PCR fragments amplified from R6M3 yielded the R6\textsuperscript{2x}, spr1704\textsuperscript{-M3} transformants which had a two-fold increase in cefotaxime MIC (Table 5). The level of resistance of R6M3 can thus be fully explained by the acquisition of mutations in \textit{php2x}, the mutation in the ABC transporter ATP-binding subunit spr1704, the mutation in the spr1333 peptidoglycan GlcNAc deacetylase, and finally by the mutation in the spr0981 glycosyltransferase.
Discussion

The strategy of reconstructing resistance by whole genome transformation is a useful approach for narrowing down the list of candidate genes implicated in resistance because we can select the recombination event directly by antibiotics. We used the combination of resistance reconstruction by transformation and genome sequencing in order to find novel mechanisms of resistance to cefotaxime. Modifications in PBPs are important contributors to resistance to β-lactam antibiotics in clinical isolates of *S. pneumoniae* and this was also the case in our panel of mutants. In clinical isolates, mosaic genes encoding PBP variants of lower antibiotic binding affinities are mainly the result of intra- and interspecies gene transfer events involving related streptococcal species.(Chambers 1999; Hakenbeck et al. 1999) This phenomenon can be mimicked *in vitro* by the acquisition of point mutations at relevant positions within the transpeptidase domain of PBPs of mutants resistant to β-lactams, as observed here for our *S. pneumoniae* cefotaxime resistant mutants. PBP2x is one of the primary cefotaxime resistance determinants in *S. pneumoniae* and the acquisition of a low-affinity PBP2x is a prerequisite for higher levels of resistance. Four *pbp2x* mutations were selected in each of our mutants (Table 2), of which the M289T and Q552E substitutions were showed in at least 2 mutants. The Q552E substitution acquired by both R6M1 and R6M3 mutants is a major determinant of resistance to β-lactam antibiotics.(Mouz et al. 1998; Mouz et al. 1999; Pernot et al. 2004; Fani et al. 2011) Indeed, this mutation is located in the vicinity of the third catalytic motif of the active site of PBP2x,(Chesnel et al. 2002) and the addition of a negative charge at position 552 of the protein was shown to decrease the β-lactams acylation efficiency of the protein.(Mouz et al. 1999; Maurer et al. 2008) The M289T substitution acquired by both R6M1 and R6M2 is located away from the catalytic cleft and has probably a more secondary role in resistance by having only minor effects with respect to the active site of the protein or by having a role only in the background of other mutations.(Maurer et al. 2008)

Other PBP2x mutations that have been specifically selected in at least one of our mutants include the T550A substitution just after the conserved KSG motif uniquely
acquired by the R6M2 mutant, the G422C mutation selected in R6M1 and the G601V and G597D substitutions observed specifically in R6M3. The T550A substitution is an important contributor to low–level cefotaxime resistance in laboratory mutants and clinical isolates of *S. pneumoniae*, (Coffey et al. 1995; Grebe and Hakenbeck 1996; Sifaoui et al. 1996; Krauss and Hakenbeck 1997; Asahi et al. 1999; Sanbongi et al. 2004) and was the sole mutation transferred to the T1M2 transformant (cefotaxime MIC 0.125mg/L) (Table 4). Threonine 550 is located close to the active site of PBP2x, where it is in direct contact with cephalosporins, (Mouz et al. 1999) and the loss of hydrogen bonding between the threonine at position 550 and cefotaxime was shown to account for resistance. (Gordon et al. 2000) The G422C mutation observed in R6M1 is positioned on the surface of the transpeptidase domain where it is distant from the active site of PBP2x, (Maurer et al. 2008) and its role in resistance is thus probably topological. Likewise, the G601V and G597D mutations acquired by R6M3 are facing similar directions on the C-terminal region of helix α11 of PBP2x and are probably affecting its active site by changing the regional topology of the catalytic cleft due to the introduction of bulkier side chains. (Maurer et al. 2008) Other PBP2x mutations identified in our cefotaxime-resistant strains are expected to have more indirect roles in resistance, like the G408E, P522L, Y524C and M527T substitutions.

Due to the intrinsically low affinity of PBP2a to β-lactam antibiotics, (Du Plessis et al. 2000; Zhao et al. 2000) substitutions in PBP2a were not thought to contribute importantly to resistance. However, an A463E substitution close to the SLN catalytic motif of PBP2a has been selected in both R6M1 and R6M2 mutants and their transformation into *S. pneumoniae* R6\(^{2x-M1}\) and R6\(^{2x-M2}\) conferred additional cefotaxime resistance (Table 5). This is consistent with other studies reporting the presence of mutation in PBP2a in penicillin-resistant and cefotaxime-resistant *S. pneumoniae* clinical and laboratory isolates. (Reichmann et al. 1996; Sanbongi et al. 2004; Smith et al. 2005b) Finally, a T242K mutation located adjacent to the KTG box in the low molecular weight PBP3 was observed in R6M2 and its transformation into R6\(^{2x2a-M2}\) increased cefotaxime resistance (Table 5). Although the precise role of this mutation in resistance remains to be established, it is intriguing that a similar T242I change had
previously been detected in *S. pneumoniae* laboratory mutants resistant to cefotaxime. (Krauss and Hakenbeck 1997)

While mutations in PBPs were contributed to cefotaxime resistance in every mutant, non-PBP resistance determinants were highlighted in this study. The gene spr1333 coding for the peptidoglycan GlcNAc deacetylase PdgA, (Vollmer and Tomasz 2000) was mutated in both R6M2 and R6M3 mutants (Table 2). In *S. pneumoniae*, a significant proportion of the GlcNAc residues within the cell wall are N-deacetylated and the inactivation of *pdgA* was shown to produce cells expressing fully N-acetylated glycans that are more sensitive to lysozyme, (Vollmer and Tomasz 2000) in addition to be less virulent in an intraperitoneal mouse model. (Vollmer and Tomasz 2002) While the precise role of PdgA in β-lactam resistance remains to be clarified, it is intriguing that alterations in the level of O-acetylation of peptidoglycan muropeptides influence resistance to penicillin in *S. pneumoniae*. (Crisostomo et al. 2006) A *pdgA* point mutation implicated in resistance to β-lactams has also been described upon transformation of *S. pneumoniae* R6 with gDNA derived from a penicillin-resistant clinical isolate, (Tait-Kamradt et al. 2009) where a H266Y substitution was shown to confer a two-fold increase in penicillin resistance. (Tait-Kamradt et al. 2009) A doubling of resistance for cefotaxime was also experimentally confirmed in our study, which was the result of the *pdgA* non-sense mutation of R6M2. The H266Y mutation also required a background of altered PBPs to confer β-lactam resistance, (Tait-Kamradt et al. 2009) and this could explain the tardy selection of the inactivating mutations within the *pdgA* gene of R6M2 (Table 4) and R6M3 (Table 6).

SrtA is a highly conserved transpeptidase responsible for the covalent attachment of LPXTG-motif-containing proteins to the cell wall. (Clancy et al. 2010) In *S. pneumoniae*, SrtA was shown to be involved in adhesion to pharyngeal cells in vitro, (Kharat and Tomasz 2003) and in animal models. (Chen et al. 2005) While the nonsense mutation within the *srtA* gene of R6M1 was able to confer resistance to cefotaxime when expressed in a *S. pneumoniae* WT background, it proved impossible to introduce this mutation into *S. pneumoniae* R6^{2x2a-M1}. The stop codon at position 121
of SrtA only occurred in the last-level transformant T3M1 however, while other mutations had already been selected, and it is possible that a specific genetic background is required for SrtA to confer resistance in the presence of altered PBPs. Consistent with this hypothesis, replacing the mutated spr1098 allele from T3M1 by a functional WT version of the gene caused a four-fold sensitization to cefotaxime. Interestingly, a decreased expression of sortase-encoding genes as well as several surface protein-encoding genes has been observed in vancomycin-resistant *Staphylococcus aureus* isolates, (McAleese et al. 2006) suggesting a possibly more global role for sortases in the response to cell wall-induced stresses.

The mutations in *strA* and *pdgA* could respectively explain cefotaxime resistance of R6M1 and R6M2 that was not due to mutations within PBPs but transformation experiments revealed that an additional mutation in the glycosyltransferase CpoA (spr0981) was required to explain the higher levels of resistance of R6M3 (Table 5). While the role in resistance for altered alleles of *cpoA* was thought to be restricted to penicillins, (Grebe et al. 1997; Hakenbeck et al. 1999) the D213Y mutation in CpoA from R6M3 clearly conferred a four-fold increase in cefotaxime resistance (Table 5). Prokaryotic CpoA homologues are involved in teichoic acid or LPS biosynthesis and the *S. pneumoniae* CpoA was shown to act as a lipid glycosyltransferase by transferring a galactose moiety to monoglucosyl-diacylglycerol (α-MGlcdAG) to produce galactosyl-glucosyldiacylglycerol (GalGlcDAG), the main glycolipid in *S. pneumoniae*. (Edman et al. 2003) It is thus possible that altered *cpoA* alleles may result in a modified synthesis of the polymer, thereby altering indirectly the property of the cell envelope and modulating the activity of cell wall antibiotics.

The genome sequence of R6M3 revealed that the ABC protein gene spr1704 was also mutated in this mutant. The increase in cefotaxime MIC following introduction of the point mutation in spr1704 confirmed the association of this ABC protein gene in resistance to cefotaxime. The genome of *S. pneumoniae* encodes several ABC proteins, some of which were shown to be involved in drug resistance. (Robertson et al. 2005; Soualhine et al. 2005; Marrer et al. 2006; Feng et al. 2009; Garvey et al. 2010)
Resistance to β-lactam antibiotics has been extensively studied in *S. pneumoniae* and while the mosaic *pbp* genes are well established as primary resistance mechanisms, considerable evidence suggested that other genes may be producing resistance. The whole genome transformation and sequencing strategy used here proved useful for identifying some of these shared and lineage-specific cefotaxime resistance determinants.
Acknowledgments

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Transparency declaration

None to declare.
Figure Legends

Figure S1: A. Stepwise selection of the *S. pneumoniae* R6M1, R6M2 and R6M2 CTX-resistant mutants. The five steps of selection on CTX gradient agar plates involved in the selection of each mutant line are represented by arrows. The 1M3 and 2M3 strains discussed in the text are referring to the third and the penultimate steps of selection of R6M3, respectively. B. Reconstruction of CTX resistance in *Streptococcus pneumoniae* by whole genome transformation (WGT). Genomic DNA derived from the *S. pneumoniae* CTX-resistant mutants R6M1 (left), R6M2 (middle) and R6M3 (right) were used for the serial transformation of *S. pneumoniae* R6 wild-type recipients. Three rounds of WGT were required to fully reconstruct the level of resistance of R6M1 and R6M2. For R6M3, we could never increase resistance to levels higher than 0.25mg/L CTX. Dashed lines indicate the WGT steps and point toward the recipient lines. Full lines point toward the transformant resulting from each WGT steps. The genomes of the strains boxed in red have been sequenced. CTX, cefotaxime; gDNA, genomic DNA; MIC, minimum inhibitory concentration; WGT, whole genome transformation.
References


47. Grebe T, Hakenbeck R. Penicillin-binding proteins 2b and 2x of Streptococcus pneumoniae are primary resistance determinants for different classes of beta-lactam antibiotics. *Antimicrob Agents Chemother* 1996; **40**: 829-34.


Authors’ contributions

FF and MO designed the study. FF and MCB performed the experiments and analyzed the data. FF drafted the manuscript. PL helped analyzing the data, revised the manuscript and provided critical comments. All authors approved the final version of the manuscript.
Table 1. Susceptibility levels of \textit{S. pneumoniae} isolates.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>R6-WT</th>
<th>R6M1</th>
<th>R6M2</th>
<th>R6M3</th>
<th>T3M1*</th>
<th>T3M2*</th>
<th>T1M3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX</td>
<td>0.023</td>
<td>4.0</td>
<td>4.0</td>
<td>8.0</td>
<td>4.0</td>
<td>4.0</td>
<td>0.25</td>
</tr>
<tr>
<td>PG</td>
<td>0.023</td>
<td>0.125</td>
<td>0.06</td>
<td>0.25</td>
<td>0.125</td>
<td>0.06</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Results are the average of at least three independent measurements. WT, wild-type; CTX, Cefotaxime; PG, Penicillin G.
*R6 cells have been transformed with the DNA of R6M1, R6M2 and R6M3 respectively.
<table>
<thead>
<tr>
<th>Locus name</th>
<th>Putative identification</th>
<th>S. pneumoniae strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R6M1</td>
</tr>
<tr>
<td><strong>spr002</strong></td>
<td>DNA biosynthesis, sliding clamp subunit, required for high processivity; DNA polymerase III beta-subunit</td>
<td>A1051G</td>
</tr>
<tr>
<td><strong>spr0046</strong></td>
<td>Phospho-6-oxoformylglycinamide synthetase</td>
<td></td>
</tr>
<tr>
<td><strong>spr0180</strong></td>
<td>Cardiolipin synthase (cls)</td>
<td></td>
</tr>
<tr>
<td><strong>spr0182</strong></td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td><strong>spr0294</strong></td>
<td>Phosphotransferase system sugar-specific EI component</td>
<td></td>
</tr>
<tr>
<td><strong>spr0303</strong></td>
<td>Cell division protein</td>
<td></td>
</tr>
<tr>
<td><strong>spr0304</strong></td>
<td>Penicillin-binding protein 2X</td>
<td>T866C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G1264T</td>
</tr>
<tr>
<td><strong>spr046</strong></td>
<td>Type I restriction enzyme EcoKI specificity protein (S protein)</td>
<td></td>
</tr>
<tr>
<td><strong>spr0526</strong></td>
<td>ABC transporter membrane-spanning permease - Pep export</td>
<td></td>
</tr>
<tr>
<td><strong>spr0551</strong></td>
<td>Branched-chain amino acid transport system carrier protein</td>
<td></td>
</tr>
<tr>
<td><strong>spr0652</strong></td>
<td>Conserved hypothetical protein</td>
<td></td>
</tr>
<tr>
<td><strong>spr0718</strong></td>
<td>Transposase</td>
<td></td>
</tr>
<tr>
<td><strong>spr0725</strong></td>
<td>ATP dependent protease (clpE)</td>
<td></td>
</tr>
<tr>
<td><strong>spr0776</strong></td>
<td>D-alanyl-D-alanine carboxypeptidase (PBP-3)</td>
<td></td>
</tr>
<tr>
<td><strong>spr0839</strong></td>
<td>Conserved hypothetical protein</td>
<td></td>
</tr>
<tr>
<td><strong>spr0981</strong></td>
<td>Glyceroltransferase</td>
<td></td>
</tr>
<tr>
<td><strong>spr1098</strong></td>
<td>Sortase</td>
<td></td>
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<tr>
<td><strong>spr1133</strong></td>
<td>3-isopropylmalate dehydratase small subunit, truncation</td>
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</tr>
<tr>
<td><strong>spr1120</strong></td>
<td>ABC transporter membrane spanning permease - glutamine transport</td>
<td></td>
</tr>
<tr>
<td><strong>spr1255</strong></td>
<td>ABC transporter membrane-spanning permease - phosphate transport (mrA)</td>
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<td><strong>spr1333</strong></td>
<td>Peptidoglycan GlcNAc deacetylase (PgdA)</td>
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</tr>
<tr>
<td><strong>spr1400</strong></td>
<td>Conserved hypothetical protein</td>
<td></td>
</tr>
<tr>
<td><strong>Spr1462</strong></td>
<td>Conserved hypothetical protein</td>
<td></td>
</tr>
<tr>
<td><strong>spr1656</strong></td>
<td>ABC transporter ATP-binding/membrane spanning protein - unknown substrate</td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Mutation 1</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>spr1704</td>
<td>ABC transporter ATP-binding protein - oligopeptide transport</td>
<td>C652T</td>
</tr>
<tr>
<td>spr1754</td>
<td>Autolysin (N-acetylmuramoyl-L-alanine amidase)</td>
<td></td>
</tr>
<tr>
<td>spr1777</td>
<td>DNA-dependent RNA polymerase subunit beta</td>
<td>G1246T</td>
</tr>
<tr>
<td>spr1823</td>
<td>Penicillin-binding protein 2A</td>
<td>C1388A</td>
</tr>
<tr>
<td>spr1838</td>
<td>Hypothetical protein</td>
<td>C258A</td>
</tr>
<tr>
<td>spr1870</td>
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</tr>
<tr>
<td>spr1991</td>
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<td>spr1992</td>
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<tr>
<td>spr1995</td>
<td>Choline binding protein A</td>
<td>G700T</td>
</tr>
</tbody>
</table>

Mutations are shown as nucleotide changes in the first line and the corresponding amino acid changes shown in italic on the second line. Asterix (*) and SYN indicate nonsense mutations and synonymous mutations, respectively.
Table 3. Chronological appearance of the identified mutations in R6M1 according to the levels of cefotaxime resistance in whole genome transformants.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene</th>
<th>spr0304 (pbp2x)</th>
<th>spr1823 (pbp2a)</th>
<th>spr1098</th>
<th>spr1870</th>
</tr>
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<tbody>
<tr>
<td>T1M1;CTX, 0.5</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>C337T</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>C1654G</td>
<td>C1388A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Q552E</td>
<td>A463E</td>
<td>R112C</td>
</tr>
<tr>
<td>T2M1;CTX, 1.0</td>
<td>spr0304</td>
<td>T866C G1264T</td>
<td>T1580C</td>
<td>C1654G</td>
<td>C337T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C1388A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M289T G422C M527T Q552E A463E</td>
<td></td>
<td></td>
<td>R112C</td>
</tr>
<tr>
<td>T3M1;CTX, 4.0</td>
<td>spr0304</td>
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<td>C1654G</td>
<td>G361T</td>
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<tr>
<td></td>
<td></td>
<td>M289T G422C M527T Q552E A463E G121*</td>
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<td>R112C</td>
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</tbody>
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Mutations are shown as nucleotide changes in the first line and the corresponding amino acid changes shown in italic on the second line. Asterix (*) indicates nonsense mutations.
Table 4. Chronological appearance of the identified mutations in R6M2 according to the levels of cefotaxime resistance in whole genome transformants.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Gene</th>
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<th>spr0776 (pbp3)</th>
<th>spr1333 (pdgA)</th>
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</thead>
<tbody>
<tr>
<td>T1M2;CT, 0.125</td>
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<td>A1648G T550A</td>
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<tr>
<td>T2M2;CT, 2.0</td>
<td>T866C</td>
<td>G1223A</td>
<td>A1571G A1648G T550A C1388A C725A</td>
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</tr>
<tr>
<td></td>
<td>M289T</td>
<td>G408E Y524C</td>
<td>T550A A463E T242K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3M2;CT, 4.0</td>
<td>T866C</td>
<td>G1223A</td>
<td>A1571G A1648G T550A C1388A C725A G754T</td>
<td>-</td>
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<tr>
<td></td>
<td>M289T</td>
<td>G408E Y524C</td>
<td>T550A A463E T242K E252*</td>
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</table>

Mutations are shown as nucleotide changes in the first line and the corresponding amino acid changes shown in italic on the second line. Asterix (*) indicates nonsense mutations.
Table 5. Minimal inhibitory concentrations to cefotaxime for R6 derivatives generated in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Allele</th>
<th>MIC (mg/L) CTX</th>
<th>PBP2x</th>
<th>PBP2a</th>
<th>PBP3</th>
<th>spr0981</th>
<th>spr1098</th>
<th>spr1333</th>
<th>spr1704</th>
</tr>
</thead>
<tbody>
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<td>R6</td>
<td>WT</td>
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<td>WT</td>
<td>WT</td>
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<td>WT</td>
</tr>
<tr>
<td>R6^{2x-M1}</td>
<td>M1</td>
<td>0.5</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>R6^{2x,2a-M1}</td>
<td>M1</td>
<td>1.0</td>
<td>M1</td>
<td>M1</td>
<td>M1</td>
<td>M1</td>
<td>M1</td>
<td>M1</td>
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* Results are the average of at least three independent measurements. CTX, cefotaxime; SM, streptomycin. 1M3, R6M3 with an MIC of 1.0 mg/L CTX; 2M3, R6M3 with an MIC of 2.0 mg/L CTX.
Table 6. Chronological appearance of the identified mutations in R6M3 according to the levels of cefotaxime resistance during in vitro selection.

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Mutations are shown as nucleotide changes in the first line and the corresponding amino acid changes shown in italic on the second line. Asterix (*) and SYN indicate nonsense mutations and synonymous mutations, respectively. 1M3, R6M3 with an MIC of 1.0 mg/L CTX ; 2M3, R6M3 with an MIC of 2.0 mg/L CTX.
Chapter IX. Genomic analyses of DNA transformation and penicillin resistance in *Streptococcus pneumoniae* clinical isolates.

This chapter contains a manuscript that was submitted to “Journal of Antimicrobial Chemotherapy” as a research article.

9.1 Résumé

Des altérations dans les protéines liant la pénicilline (PLP), les enzymes cibles de la famille β-lactam, sont reconnues comme mécanismes principaux de la résistance à la pénicilline chez *Streptococcus pneumoniae*. Néanmoins, des mécanismes de résistance supplémentaires ont été répertoriés. Nous avons reconstruit la résistance à la pénicilline par transformation avec l’ADN génomique provenant de trois souches cliniques résistantes à la pénicilline dans *S. pneumoniae* R6. La séquence du génome des trois transformants T2-18209, T5-1983 et T3-55938 a révélé que 16.2 kb, 82.7 kb et 137.2 kb de leurs génomes ont été remplacés par 3, 10 et 23 blocs de séquence comprenant plusieurs gènes. Le séquençage des transformants ayant le même niveau de résistance à la pénicilline retrouvé chez les souches parentales cliniques a confirmé l'importance de la mosaïque PBP2x, 2b et 1a en tant que force motrice de la résistance à la pénicilline. Un rôle dans la résistance pour PBP2a mosaïque a également été observé pour deux des souches cliniques résistants. Nous présentons également un nouveau rôle pour une alpha-amylase cytoplasmique conférant une résistance modérée à la pénicilline en présence d'altération des protéines liant à la pénicilline.
Genomic analyses of DNA transformation and penicillin resistance in \textit{Streptococcus pneumoniae} clinical isolates.

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Running title: penicillin resistance in \textit{Streptococcus pneumoniae} clinical isolates
Abstract

Alterations in penicillin-binding proteins, the target enzymes for β-lactam antibiotics, are recognized as primary penicillin resistance mechanisms in *Streptococcus pneumoniae*. Nonetheless, evidence is available for the involvement of additional resistance mechanisms. We reconstructed penicillin resistance by serial genome transformation of the DNAs derived from three penicillin resistant clinical isolates into a sensitive *S. pneumoniae* background. The genome sequence of the three transformants T2-18209, T5-1983 and T3-55938 revealed that 16.2 kb, 82.7kb and 137.2 kb of their genomes had been replaced with 3, 10 and 23 multiple-gene recombinant sequence segment (RSSs) of the respective parental clinical isolates, documenting the extent of DNA transformation between strains. Sequencing of the transformants with similar minimal inhibitory concentrations for penicillin as the parent clinical strains also confirmed the importance of mosaic PBP2x, 2b and 1a as a driving force in penicillin resistance. A role in resistance for mosaic PBP2a was also observed for two of the clinical resistant isolates. We also report a new role for a cytoplasmic alpha amylase in conferring moderate resistance to penicillin in the presence of altered penicillin-binding proteins.
Keywords: *Streptococcus pneumoniae*, Whole genome sequencing, Antibiotic resistance, Penicillin, DNA transformation
Introduction

*Streptococcus pneumoniae* is a Gram-positive pathogen responsible for serious diseases such as pneumonia, meningitis, acute otitis media and sepsis. Penicillin (PG), a β-lactam antibiotic, has long been the mainstay against pneumococcal infections but its efficacy is threatened by the rapid dissemination of PG non-susceptible clones worldwide. Resistance to β-lactam antibiotics in clinical isolates of *S. pneumoniae* is mediated by mosaic genes encoding altered penicillin binding proteins (PBPs), a family of transpeptidases and carboxypeptidases involved in peptidoglycan metabolism, with lower antibiotic binding affinities than their native versions (Chambers 1999; Hakenbeck et al. 1999). While *S. pneumoniae* contains six PBPs, variants of PBP2x, PBP2b and PBP1a are considered the most relevant for PG resistance and the acquisition of low-affinity PBP2x and PBP2b variants are a necessary first step for the acquisition of PBP1a variants to confer high-level resistance to β-lactams (Smith and Klugman 1998; du Plessis et al. 2002). Pneumococci have a dedicated system for the acquisition of exogenous DNA from the environment and the mosaic gene structure of low-affinity PBPs is the result of interspecies gene transfer events involving closely related streptococcal species (Hakenbeck et al. 1998; Chambers 1999). Nonetheless, the varying degree of resistance observed among non-susceptible clinical isolates suggests that resistance involves other complex and multifactorial processes and the presence of other non-PBP contributors have indeed been reported. For example, the cell wall of penicillin non-susceptible isolates is often highly enriched in branched chain muropeptides, a phenomenon that has been linked to mosaic alleles of the murM gene (Filipe and Tomasz 2000; Smith and Klugman 2001). Furthermore, mutations in a peptidoglycan N-acetylglucosamine (GlcNAc) deacetylase (Tait-Kamradt et al. 2009), a peptidoglycan O-acetyltransferase (Crisostomo et al. 2006), a putative glycosyltransferase (Grebe and Hakenbeck 1996), a serine threonine kinase (Dias et al. 2009), a histidine protein kinase part of a two-component signal transducing system (Guenzi et al. 1994), or in a phosphate ABC transporter (Soualhine et al. 2005) have all been implicated in resistance to β-lactams. Finally, the selection of a nonsense mutation in a putative iron permease in penicillin-resistant *S. pneumoniae* has recently been
shown to increase tolerance to bactericidal antibiotics, including penicillin, by preventing the accumulation of reactive oxygen species (Fani et al. 2011).

The identification of resistance mechanisms in *S. pneumoniae* clinical isolates is complicated by the substantial polymorphisms between field isolates (Hiller et al. 2007). However, phenotypic reconstruction by whole genome transformation (WGT) of antibiotic-sensitive *S. pneumoniae* of known genetic backgrounds with genomic DNA derived from clinical isolates, coupled to next generation sequencing (NGS) of antibiotic resistant transformants, constitute a powerful strategy for pinpointing determinants of resistance at the genome level (Tait-Kamradt et al. 2009; Billal et al. 2011). Here, we succeeded in reconstructing the resistance of three *S. pneumoniae* penicillin non-susceptible clinical isolates into the penicillin sensitive *S. pneumoniae* R6 by genome transformation. Sequencing of these transformants indicated the extent of DNA transformation between strains, confirmed the role of mosaic PBPs in resistance, and allowed finding a new gene involved in resistance.
Materials and Methods

Bacterial strains, culture conditions and MIC determination

Pneumococci were grown in brain heart infusion broth (BHI, Difco), or in blood agar containing 5% defibrinated sheep’s blood as described previously (Munoz et al. 1992). Cultures were incubated for 16 to 24 hours in a 5% CO₂ atmosphere at 35°C. The T2-18209, T3-55938 and T5-1983 penicillin-resistant transformants were generated in S. pneumoniae R6 (Hoskins et al. 2001). The minimum inhibitory concentration (MIC) to penicillin was determined with E-test strips (AB Biodisk) on Müller-Hinton agar plates supplemented with 5% sheep blood using manufacturer’s instructions. The MICs were further confirmed by the microdilution method according to the Clinical Laboratory Standards Institute (CLSI) guidelines.

Transformation experiments

High molecular weight genomic DNA (gDNA) was extracted from the penicillin-resistant clinical isolates using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions. The integrity of gDNA prior to whole genome transformation was assessed by agarose gel electrophoresis. In initial experiments, high molecular weight gDNA from clinical isolates CCRI-18209, 55938 and CCRI-1983 were used to serially transform S. pneumoniae R6. The selection of transformants was done by increasing concentrations of PG. Pneumococci were rendered competent by culturing at 35°C in C+Y medium at pH 6.8 (Tomasz and Hotchkiss 1964) until the onset of exponential phase. The cells were then concentrated tenfold and resuspended in C+Y medium at pH 7.8 supplemented with 15% glycerol and frozen at -80°C. For transformation, competent cells were thawed on ice, diluted ten times with C+Y medium at pH 7.8 and stimulated with 2μg/ml of competence stimulating peptide 1 (csp-1) for 15 minutes at 35°C under a 5% CO₂ atmosphere. gDNA or PCR products were added to a final concentration of 2μg/ml and the cultures were incubated for 1 hour at 30°C, followed by 1 hour at 35°C under a 5% CO₂ atmosphere. The cultures were then plated on CAT agar with the appropriate concentration of PG and incubated for 48 hours at 35°C under a 5% CO₂ atmosphere.
Whole Genome Sequencing

gDNAs were prepared from mid-log phase S. pneumoniae cultures using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer’s instructions. The genomes of the T2-18209, T3-55938 and T5-1983 transformants were sequenced using the 454 Life Sciences (Roche) GS-FLX Titanium system (McGill University Genome Quebec Innovation Center) which generated a genome assembly of 33× coverage, with 97% of the reads assembled into 48 and 77 large contigs, respectively. Intermediate-level transformants derived from strain CCRI-1983 were sequenced using an Illumina HiSeq1000 system (Centre de Recherche en Infectiologie of Université Laval) using a 101-nucleotides paired-end reads protocol which generated a genome assembly of 27x coverage. Intermediate-level transformants derived from strains CCRI-18209 and 55938 were sequenced using an Illumina MiSeq system (Centre de Recherche en Infectiologie of Université Laval) using a 250-nucleotides paired-end reads protocol. Sequence reads from each strain were aligned to the genome of S. pneumoniae R6 (Hoskins et al. 2001) using the software bwa (bwa aln, version 0.5.9) with default parameters (Li and Durbin 2009). The maximum number of mismatches was 4, the seed length was 32 and 2 mismatches were allowed within the seed. The detection of single nucleotide polymorphisms was performed using samtools (version 0.1.18), bcftools (distributed with samtools) and vcfutils.pl (distributed with samtools)(Li et al. 2009), with a minimum of three reads to call a potential variation prior to further analysis. The sequence data are available at the EMBL European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the study accession number ERP001840; samples ERS225580 and ERS179073 for S. pneumoniae T1-18209 and T2-18209, respectively; samples ERS225581, ERS225582 and ERS179074 for S. pneumoniae T1- to T3-55938, respectively; samples ERS225576, ERS225577, ERS225578, ERS225579 and ERS179072 for S. pneumoniae T1- to T5-1983, respectively. Mutations deduced from massively parallel sequencing were confirmed by PCR amplification and Sanger sequencing.
Results

In order to identify the mutations linked to PG resistance of our *S. pneumoniae* clinical isolates, genomic DNAs derived from CCRI-18209, CCRI-1983 and 55938 were used for reconstructing resistance by whole genome transformation into *S. pneumoniae* R6. The selection of transformants under PG pressure favors the transfer of regions involved in resistance and hence facilitates their discrimination from natural polymorphism occurring in field isolates. A total of two, three, and five rounds of transformation were required to fully reconstruct the PG resistance levels of CCRI-18209, 55938 and CCRI-1983, respectively (Table 1).

The genome sequence of the fully resistant transformants T2-18209, T3-55938 and T5-1983 and of every intermediate-step transformants was established to determine the set of mutations selectively transferred while reconstructing PG resistance. Alignment of the *S. pneumoniae* R6 wild-type (WT) and T2-18209 genome sequences identified a total of 1660 SNPs distributed over 5 recombinant sequence segments (RSSs) spanning a total of ~16.2 kb in the genome of T2-18209 (Fig. 1A). RSSs were defined as contiguous runs of polymorphic sites bounded by recipient-specific alleles in the *S. pneumoniae* R6 transformants. Three of the T2-18209 RSSs included more than one gene and are referred to as multi-gene RSSs, of which two contained mosaic PBPs genes *(pbp2x and pbp2b)* transferred from CCRI-18209 as part of distinct RSSs (Fig. 1A). The size of these three multi-gene RSSs varied from 1.6 kb to 9.9 kb (mean of 4.7 kb). The two others RSSs of T2-18209 were less than 2.3 kb and uniquely carried the *pbp1a* and *spr1916* genes, respectively (Fig. 1A). Alignment of the *S. pneumoniae* R6 WT and T5-1983 genomes revealed a total of 1502 SNPs organized as 20 RSSs ranging in size from less than 1 kb to 19.4 kb and spanning a total of ~ 82.7 kb in T5-1983 (Fig. 1B). Ten of the T5-1983 RSSs had a mean size of 1.2 kb (ranging from 0.3 to 2.4 kb) and were composed of unique genes while ten others were found as significantly larger (p<0.001) multi-gene RSSs with a mean size of 8.3 kb (ranging from 2.4 to 19.4 kb) (Fig. 1B). Mosaic *pbp2x, pbp1a, pbp2b* and *pbp2a* were transferred from CCRI-1983 into T5-1983 as part of multi-gene RSSs. Finally, the alignment of the T3-55938 genome sequence to *S. pneumoniae* R6 revealed 812 donor
SNPs clustered into 37 RSSs (23 multi-genes and 14 single genes) spanning a total of 137.2 kb (Fig. 1C). With a mean size of 6 kb (ranging from 1.4 to 15.5 kb), the multi-genes RSSs were significantly larger (p<0.001) than the other RSSs which had a mean size of 1.4 kb (ranging from 0.2 to 2.7 kb). Similarly to the T5-1983 transformant, T3-55938 acquired mosaic *php2x*, *php1a* and *php2b* genes as part of multi-gene RSSs, but this time the mosaic version of *php2a* was acquired as part of a single gene recombinant unit (Fig. 1C).

Transformation experiments with PBP genes amplified from the T2-18209, T5-1983 and T3-55938 were conducted with *S. pneumoniae* R6 WT recipients to assess the contribution of the different mosaic PBPs to PG resistance. While resistance mediated by mosaic PBP1a can only be detected in the presence of low-affinity PBP2x and/or PBP2b, the latter two genes confer resistance when introduced in WT cells and are thus considered the primary targets of β-lactams (Hakenbeck et al. 1998; Smith and Klugman 2005; Stanhope et al. 2008). The acquisition of low-affinity PBP2x and PBP2b variants was shown to be prerequisites for PBP1a variants to confer high-level resistance to β-lactams (Smith and Klugman 1998; du Plessis et al. 2002) and in vitro penicillin mutants mutations in *php2x* preceded mutations in *php2b* (Fani et al. 2011). Consequently, *php2x* was first amplified from genomic DNAs derived from T2-18209, T5-1983 and T3-55938 using the primers listed in Table S1 and the PCR products transformed into *S. pneumoniae* R6 WT. The independent selection of transformants with a PG concentration of 0.06 μg/ml enabled recovering the transformants R6\(^{2x-18209}\), R6\(^{2x-1983}\) and R6\(^{2x-55938}\) which had a two- to four-fold increase in PG MIC (0.06, 0.125 and 0.06μg/ml, respectively) compared to *S. pneumoniae* R6 WT (Table 2). The targeted sequencing of *php2x* from R6\(^{2x-18209}\), R6\(^{2x-1983}\) and R6\(^{2x-55938}\) confirmed that every *php2x* mutations from T2-18209, T5-1983 and T3-55938 were transferred. A second round of transformation using R6\(^{2x-18209}\), R6\(^{2x-1983}\) and R6\(^{2x-55938}\) as recipients and PCR products covering the mutated *php2b* of T2-18209, T5-1983 and T3-55938 further increased the PG MIC four times, producing the R6\(^{2x, 2b-18209}\), R6\(^{2x, 2b-1983}\) and R6\(^{2x, 2b-55938}\) transformants with PG MICs of 0.25, 0.5 and 0.25 μg/ml, respectively (Table 2). In a third round of transformation, the acquisition of a mosaic
*pbp1a* by R6^{2x, 2b-18209}, R6^{2x, 2b-1983}, and R6^{2x, 2b-55938} enabled increasing the PG MIC to 1-2 μg/ml in the transformants (Table 2). Since these MIC values remained lower than those observed in the parental transformants or clinical isolates (Table 1), other mutations than in *pbp2x*, *pbp2b* and *pbp1a* present in the last step transformants should be involved in PG resistance.

While a role for PBP2a alterations in PG resistance has only been observed sporadically (Du Plessis et al. 2000; Zhao et al. 2000; Carapito et al. 2006), both T3-55938 and T5-1983 where found to harbor altered versions of *pbp2a* (Fig. 1B and C). In the case of T3-55938, the transformation of R6^{2x, 2b, 1a-55938} recipients with a PCR product covering *pbp2a* from T3-55938 yielded the R6^{2x, 2b, 1a, 2a-55938} transformants with a PG MIC of 4 μg/ml (Table 2). Thus, the resistance level of the T3-55938 transformant (and therefore of its parent mutant 55938) can be entirely explained by the acquisition of the four mosaic PBP genes (Table 2), with the genome sequences of T1-, T2- and T3-55938 revealing the order of acquisition of these variant alleles being the 3' parts of *pbp2x* and *pbp2b* occurring first, followed by *pbp1a* and the 5' parts of *pbp2x* and *pbp2b*, and lastly *pbp2a* (Fig. 1C). This also suggests that the many other RSSs acquired during the three rounds of 55938 gDNA transformation (Fig. 1C) either have a more subtle role in resistance or represent bystander events.

The acquisition of a mosaic version of *pbp2a* to produce R6^{2x, 2b, 1a, 2a-1983} displayed a PG MIC of 2.0μg/ml however (Table 2), which is four times less than the last transformant or the CCRI-1983 clinical isolate (Table 1). In order to find additional candidate genes with possible role in PG resistance in this transformant, we also determined the order of appearance of the mutations identified in the series of transformants derived from CCRI-1983 by sequencing the genomes of T1-1983, T2-1983, T3-1983 and T4-1983. The only mutations transferred during the first round of transformation giving rise to T1-1983 with a PG MIC of 0.06μg/ml covered the 5’ part of the *pbp2x* gene (Fig. 1B). This is half the resistance level conferred by the full set of *pbp2x* mutations from T5-1983 (Table 1), but the full set of *pbp2x* mutations was only acquired at the fifth and last step of transformation (Fig. 1B). The acquisition of a
multi-gene RSS carrying a mosaic version of *php2b* by the T2-1983 transformants along with two single-gene RSSs (Fig. 1B) further increased the PG MIC four times to 0.25 μg/ml (Table 1). Given that transforming an altered version of *php2b* in a background of mosaic *php2x* also confers a fourfold increase in PG MIC (Table 2), the difference in PG MIC between T1-1983 and T2-1983 is likely explained by the acquisition of a mosaic *php2b*. The biggest increase in MIC levels occurred after the third round of transformation, with an eight-fold increase in PG resistance from 0.25 to 2μg/ml (Table 1). The genome sequences of T2-1983 and T3-1983 revealed that they only differed by the acquisition of a multi-gene RSS carrying *php2a* along with the spr1824 and 1825 genes and another carrying the genes spr1238 and spr1239 (Fig. 1B) by T3-1983. Apart from the mosaic *php2a* which was shown to increase PG MIC to 1μg/ml when transformed into T2-1983 (Table 2), the only other gene which could be implicated in resistance to explain the PG MIC of T3-1983 is the spr1239 gene coding for an alpha-amylase with a non-sense mutation acquired from CCRI-1983. Indeed, the transformation of a PCR product covering spr1239 from T5-1983 in *S. pneumoniae* T2-1983<sup>2a-1983</sup> increased the PG MIC to the same level as T3-1983, from 1μg/ml for T2-1983<sup>2a-1983</sup> to 2μg/ml in T2-1983<sup>2a, spr1239-1983</sup> (Table 2). Despite several attempts it proved impossible to transform the same spr1239 PCR products in R6<sup>2x, 2b, 1a, 2a-1983</sup> recipients however, and the presence of altered Pbp1a might thus preclude the selection of the non-sense mutation in spr1239 (data not shown). Finally, the consecutive doubling in PG MICs observed during the fourth and last transformation steps is most probably due to the acquisition of a mosaic *php1a* by T4-1983 (Table 2 and Fig. 1B) and of a the complete set of *php2x* mutations by T5-1983 (Fig. 1B).

In the case of the T2-18209 transformant we could not identify additional PG resistance mechanisms among the RSSs transferred from CCRI- 18209 that could further explain the level of resistance of this clinical isolate besides the mosaic PBPs. Alternatively, it cannot be excluded that PBPs may contribute differently to resistance depending on the genomic context.
Discussion

We report the sequencing of *S. pneumoniae* R6 transformants generated while reconstructing the PG resistance phenotype from three *S. pneumoniae* clinical isolates and the characterization of the genetic determinants defining their resistance. Analysis of sequencing results showed that the three transformants, T2-18209, T5-1983 and T3-55938 had replaced 16.2 kb, 82.7 kb and 137.2 kb of their genomes with 3, 10 and 23 RSSs, respectively. The length of these 36 RSSs ranged from 1.4 to 19.4 kb, with an average size of 6.5 kb. These lengths are similar to those reported from analysis of naturally occurring recombination tracts observed in *Neisseria meningitides* (5.1 kb) at specific loci (Linz et al. 2000) and in *H. influenza* (8.1 kb) (Mell et al. 2011), but larger than the 2 kb found in a recent study describing the interspecies transformation of *S. pneumoniae* with high-level penicillin resistant *S. mitis* B6 DNA (Sauerbier et al. 2012). Although the transfer of smaller RSSs containing partial gene segments also occurred in our transformants, these were significantly less responsible for the PG resistance phenotype than multi-gene RSSs. Indeed, while a role in PG resistance was confirmed for 10 out of the 36 (27%) multi-gene RSSs identified in our series of transformants, only two (7%) of the 27 single-gene RSSs were associated with resistance. This is in agreement with the recent high resolution analysis of pneumococcal transformation that revealed a smaller size for bystander recombination fragments (mean of 2.3 kb) compared to recombination fragments responsible for the selected phenotype (Croucher et al. 2012).

Resistance to β-lactam antibiotics in *S. pneumoniae* clinical isolates occurs through the acquisition of mosaic genes encoding altered PBPs resulting from recombination events between PBP alleles within or across streptococcal species (Chambers 1999; Hakenbeck et al. 1999). *S. pneumoniae* contains six PBPs but only variants of PBP2x, PBP2b and PBP1a are frequently described in resistant clinical isolates. Not surprisingly, our three *S. pneumoniae* clinical isolates non-susceptible to PG contained mosaic PBP alleles whose contribution to PG resistance were confirmed by whole genome and targeted transformation into the PG susceptible *S. pneumoniae* R6. The acquisition of low-affinity PBP2x and PBP2b variants was shown to be a prerequisite
for PBP1a variants to confer high-level resistance to β-lactams (Hakenbeck et al. 1998; Smith et al. 2005b; Stanhope et al. 2008) and mutations within *pbp1a* were indeed found to have transferred after *pbp2x* and *pbp2b* mutations, as demonstrated by the targeted sequencing of the series of transformants derived from CCRI-1983. Our whole genome transformation scheme pinpointed several SNPs outside the PBPs. These other mutations may have a direct role in resistance or may have compensatory roles or may be only bystander mutations.

Two of our clinical isolates were found to harbor *pbp2a* alleles contributing to PG resistance. PBP2a was shown to have a relatively low affinity for penicillin compared with other PBPs and it has been suggested that PBP1a mutations might therefore be selected before PBP2a in resistance (Zhao et al. 2000). Nonetheless, *pbp2a* mutations from CCRI-1983 were found to have transferred at an earlier step (T2-1983) than *pbp1a* mutations (T4-1983) while reconstructing resistance by WGT. This suggests the combination of *pbp2a* mutations along with the mutations within *spr1239* (α-amylase) acquired from a co-transferred RSS by T3-1983 might supersede the benefit of *pbp1a* mutations which were only transferred at a later step. Indeed, the biggest increase in PG MIC occurred while selecting for T3-1983 transformants and was shown to solely result from the acquisition of altered versions of *pbp2a* and *spr1239*. Our failed attempts to transform *S. pneumoniae* R6*2x, 2b, 1a, 2a-1983* recipients with PCR products of *spr1239* derived from T5-1983 is suggesting that other allelic polymorphisms transferred to T3-1983 could have a more indirect role in resistance and that the recombination events might have to follow a specific order, with the presence of mosaic *pbp1a* putatively interfering with the acquisition of an altered *spr1239*. The *spr1239* gene codes for a cytoplasmic alpha-amylase, an enzyme involved in the breakdown of carbohydrates by hydrolyzing alpha bonds of large polysaccharides like starch and glycogen for their use as a carbon source. It is part of the sixteen *S. pneumoniae* genes putatively encoding proteins involved in the metabolism of α-glucans (Abbott et al. 2010), of which six (including *spr1239*) have been identified to be necessary for the full virulence of the bacterium by signature-tagged mutagenesis (Hava and Camilli 2002). Given that *S. pneumoniae* also have numerous extracellular
enzyme systems that allow the metabolism of a large number of sugars that can flow directly into the glycolytic pathway (Tettelin et al. 2001), the precise role in PG resistance for the non-sense mutation in the spr1239 alpha-amylase will require further investigations.

In summary, this study has allowed to further study DNA transformation at the genome scale level. It has also further confirmed the role of PBPs in resistance, the sequential order of PBPs mutations and potentially a greater role for PBP2a. Finally this study has highlighted one alpha-amylase as a new marker of clinical penicillin resistance.
References


Hakenbeck, R., A. Konig, et al. (1998). "Acquisition of five high-Mr penicillin-binding protein variants during transfer of high-level beta-lactam resistance from..."


Figure legends

**Figure 1.** Scaled representation of the *S. pneumoniae* R6 genome showing recombinant sequence segments acquired from CCRI-18209 (A), CCRI-1983 (B), and 55938 (C). The background genome is shown as a grey circle. The inner circle indicates chromosomal positions in a clockwise fashion. Recombinant sequence segments are shown as coloured bands according to the level of transformation they were acquired. White bands with white labels in filled black-boxes, T1 transformants; Black bands and labels, T2 transformants; Green bands and labels, T3 transformants; Blue bands and labels, T4 transformants; Red bands and labels, T5 transformants; Grey-boxed labels indicate recombinant sequence segments acquired as two partial segments during two distinct transformation steps. Pbp2x, spr0304; Pbp1a, spr0329; Pbp2b, spr1517; Pbp2a, spr1823. Alpha-amylase, spr1239.
Table 1. Minimal inhibitory concentrations to penicillin for the three gDNA donor *S. pneumoniae* clinical isolates, the *S. pneumoniae* R6 WT recipient and its penicillin-resistant transformants.

<table>
<thead>
<tr>
<th>Clinical isolates</th>
<th>Clinical isolates PG MIC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Recipient strain</th>
<th>Recipient PG MIC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Transformants PG MIC&lt;sup&gt;ab&lt;/sup&gt;</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCRI-18209</td>
<td>4.0</td>
<td>R6 WT</td>
<td>0.023</td>
<td>0.1</td>
</tr>
<tr>
<td>55938</td>
<td>4.0</td>
<td>R6 WT</td>
<td>0.023</td>
<td>0.125</td>
</tr>
<tr>
<td>CCRI-1983</td>
<td>8.0</td>
<td>R6 WT</td>
<td>0.023</td>
<td>0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup> MICs are expressed in μg/mL and are the average of three independent measurements.

<sup>b</sup> 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> indicate the level of transformation.

gDNA, genomic DNA; PG, penicillin G.
Table 2. Minimal inhibitory concentrations to penicillin for R6 derivatives constructed in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Alleles</th>
<th>PG MIC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>PBP2x</td>
<td>PBP2b</td>
</tr>
<tr>
<td>R6</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x,18209&lt;/sup&gt;</td>
<td>T2-18209</td>
<td>WT</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x,2b,18209&lt;/sup&gt;</td>
<td>T2-18209</td>
<td>T2-18209</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x,2b,1a,18209&lt;/sup&gt;</td>
<td>T2-18209</td>
<td>T2-18209</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x,1983&lt;/sup&gt;</td>
<td>T5-1983</td>
<td>WT</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x,2b,1983&lt;/sup&gt;</td>
<td>T5-1983</td>
<td>T5-1983</td>
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<tr>
<td>R6&lt;sup&gt;2x,2b,1a,1983&lt;/sup&gt;</td>
<td>T5-1983</td>
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<tr>
<td>R6&lt;sup&gt;2x,2b,1a,2a,1983&lt;/sup&gt;</td>
<td>T5-1983</td>
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<td>T2-1983</td>
<td>T5-1983</td>
<td>T5-1983</td>
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<tr>
<td>T2-1983&lt;sup&gt;2a,1983&lt;/sup&gt;</td>
<td>T5-1983</td>
<td>T5-1983</td>
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<tr>
<td>R6&lt;sup&gt;2x,55938&lt;/sup&gt;</td>
<td>T3-55938</td>
<td>WT</td>
</tr>
<tr>
<td>R6&lt;sup&gt;2x,2b,55938&lt;/sup&gt;</td>
<td>T3-55938</td>
<td>T3-55938</td>
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<tr>
<td>R6&lt;sup&gt;2x,2b,1a,55938&lt;/sup&gt;</td>
<td>T3-55938</td>
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<tr>
<td>R6&lt;sup&gt;2x,2b,1a,2a,55938&lt;/sup&gt;</td>
<td>T3-55938</td>
<td>T3-55938</td>
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<sup>a</sup>MICs are expressed in μg/mL and are the average of three independent measurements. PG, penicillin G.
Chapter X. General discussion

Most people in the second half of the 20th century would have believed that bacterial infections would be eradicated, or at least easily managed, by the discovery of antimicrobial agents and other means of infection control. However, due to the excessive and inappropriate use of antibiotics, there has been a gradual emergence of populations of antibiotic-resistant bacteria, which have posed a global health problem. The phenomenon of antimicrobial resistance has now reached epidemic proportion. Resistant infections are often fatal or responsible for prolonged illness, which would in turn increase the likelihood of transmission of infection. The annual cost of antibiotic-resistant infections in the US health care system has been estimated to be above $20 billions. Studies of resistance mechanisms permit the development of tools for the early recognition of resistance in infection, thereby preventing useless and often toxic chemotherapy. In addition, they can unveil intra-cellular drug targets and defence mechanisms, allowing the development of drug analogues.

The worldwide increase in the rates of resistance to antimicrobial agents among S. pneumoniae, particularly the traditional 1st-line agents such as penicillin, tetracycline and macrolides (Low et al. 2002) has raised concerns regarding the use of such agents for the treatment of community-acquired pneumonia (Bartlett et al. 2000). Global approaches have the power of revealing both primary resistance mutations and fitness-compensatory mutations involved in physiological as well as genetic adaptation of resistant isolates. For instance, transcriptomics (Guimond et al. 2003; Leprohon et al. 2006; Leprohon et al. 2009) and proteomics (Drummelsmith et al. 2003; Drummelsmith et al. 2004; Drummelsmith et al. 2007) have been successfully used in studying resistance. Thanks to such approaches, a number of resistance markers have been highlighted in S. pneumoniae (Cash et al. 1999; Ng et al. 2003; Haas et al. 2004; Soualhine et al. 2005; Cassone et al. 2006; Marrer et al. 2006).

10.1 Strain selection

In the present study, we initially needed to decide about the most appropriate strategy for the selection of resistant strains that would undergo genomic resistance
analyses. Although we believed that working with clinical isolates could lead directly to resistance mechanisms of clinical relevance, we decided to use genome sequencing of resistant bacteria selected for resistance in the laboratory. The reason for this choice was the large heterogeneity of clinical isolates, which would defy a direct comparison between sensitive and resistant clinical isolates. Selection of resistance in the in-vitro condition provides the possibility of working with strains that have identical genetic backgrounds but differing in susceptibility and resistance. Accordingly, comparison of isogenic isolates to which resistance was induced in the lab confers the unique advantage of having a higher likelihood of correlation between the identified SNPs and the resistance phenotype. The main disadvantage of working with lab strains, however, is that the identified resistance mechanism may not be clinically relevant. Nonetheless, it is possible to test whether the highlighted mutations are present in resistant clinical isolates that are available in our collections. We generated five S. pneumoniae isolates resistant to penicillin (PG) and cefotaxime (CTX) in a step by step fashion on gradient plates. In our initial analysis of β-lactams resistance, we sequenced two S. pneumoniae penicillin resistant strains and found a total of 78 mutations (26 for M1 and 52 for M2) compared to the parental strain. In view of the high number of mutations, we concentrated on six recurrent ones in both mutants, which were then prioritized for further biological validation. The role of each of these six genes in PG resistance was experimentally tested (by PCR transformation or gene inactivation). However, even under these controlled conditions, not all mutations were linked to resistance; some might have simply resulted from the replication error caused by drug stresses, or may have corresponded to compensatory fitness mutations.

A gene-by-gene approach to testing penicillin resistance proved to be time-consuming and unrewarding. Instead over the course of investigations of CTX mutants, we used a whole genomic transformation approach for the reconstruction of resistance, as detailed in Fig.10.1. This would narrow down the list of candidate genes. In this approach, we first isolated the DNA of a CTX resistant mutant (M1, M2 or M3) and transformed it into sensitive wild-type cells. Through successive transformations and antibiotic selection, we were able to reconstruct resistance. This method proved highly effective, which can be accounted for by the fact that we could select recombination
events directly using antibiotics. We succeeded to fully reconstruct resistance for many isolates. In all the tested cases, the mutations present in the mutant were efficiently transferred to the transformants. The whole genome transformation approach for reconstruction of resistance proved a useful strategy for narrowing down the list of mutations. This was the case, for example, with R6M1 and its transformant T3M1, where the number of mutations in T3M1 was reduced to 8 from 14 (in M1). However, we were not able to stop some transformants (e.g. T3M1 and T3M2) from selecting some spontaneous mutations, which was possibly the result of drug pressure.

Nor did we manage to fully reconstruct resistance by transformation in the case of one mutant (R6M3), the highest CTX MIC ever achieved being 0.25μg/ml. We decided, therefore, to sequence two of the five distinct increments required for in vitro selection of R6M3; namely, 1M3 and 2M3, which had an MIC of 1.0μg/ml and 2.0μg/ml, respectively. This turned out to be an effective method for pinpointing gene(s) responsible for the increase in the resistance level across increments that have increasing MIC’s.
Whole genome transformation and resistance reconstruction in *Streptococcus pneumoniae*. Genomic DNA extracted from the *S. pneumoniae* cefotaxime resistant mutants R6M1 and R6M2 was used for the serial transformation of *S. pneumoniae* R6 wild-type cells. T1, T2, and T3 stand for the first, second and third level transformations respectively. CTX, cefotaxime; NGS (Next Generation Sequencing); WGT (Whole Genome Transformation)

10.2 Genomic analyses of resistance

We have used whole genome sequencing (WGS) for the determination of mechanisms of resistance to penicillin and cefotaxime in *S. pneumoniae*. Initially, this was done by array-based comparative genome sequencing developed by NimbleGen ([www.nimblegen.com](http://www.nimblegen.com)), which relies on the use of tiled DNA microarray hybridizations to identify the location of SNPs, insertions, or deletions, with only the regions of the genome where alterations exist being sequenced by Sanger technology (Albert et al. 2005). Over the course of this study, new sequencing technologies known as “Next
"Generation Sequencing" (NGS) became more accessible due to their decreasing costs. NGS platforms share a common technological feature: the massively parallel sequencing of clonally amplified or single DNA molecules (Voelkerding et al. 2009). They revolutionized the sequencing of microbial as well as larger genomes thanks to their high-through capacity as well as the accuracy of the data they generated as compared to those produced by comparative genome hybridization (CGH or CGS). Two of such technologies to which we soon switched were 454 GS FLX sequencing and the Illumina-Genome Analyzer. Table 10.1 compares the features of these two technologies. Although both technologies have been successfully used for re-sequencing (Voelkerding et al. 2009), they have comparative advantages and drawbacks.

For a start, 454 GS FLX sequencing has a longer read length, which facilitates de novo assembly of genomes. In addition, although this technology provides accurate nucleotide haplotype information over a range of several hundred base pairs, its accuracy decreases when determining the homopolymers of higher than 3 to 4 bases (Margulies et al. 2005; Huse et al. 2007). Illumina platform, on the other hand, generates short read lengths of 36 bases, with its overall sequencing output being above 1 billion base pairs per run, i.e. two times higher than that of the 454 technology. A technical concern with this technology, however, is that its base accuracy decreases with increasing read length (Voelkerding et al. 2009).

Table 10.1. Comparison of NGS platforms

<table>
<thead>
<tr>
<th></th>
<th>Sanger</th>
<th>Roche</th>
<th>Illumina</th>
</tr>
</thead>
<tbody>
<tr>
<td>454 GS FLX</td>
<td>Dye terminator</td>
<td>Pyrosequencing</td>
<td>Reversible dye terminators</td>
</tr>
<tr>
<td>Read lengths</td>
<td>800bp</td>
<td>400bp</td>
<td>36</td>
</tr>
<tr>
<td>Sequencing run time</td>
<td>3h</td>
<td>10h</td>
<td>2.5 days</td>
</tr>
</tbody>
</table>
Total bases per run | 800bps | 500Mb | 1.5Gb

10.3 Penicillin-binding proteins (PBPs) and β-lactam resistance

Since the discovery of PBPs as targets of the β-lactams, considerable data have become available on point mutations and their role in resistance to β-lactams of some important pathogens such as *Staphylococcus aureus*, *Enterococci* and *Streptococcus pneumoniae*. β-lactam-resistant strains of *S. pneumoniae* always contain altered versions of their PBPs that have a lower affinity for β-lactams, and which are poorly acylated by β-lactams (Hakenbeck et al. 1980; Zighelboim and Tomasz 1980).

PB2p is documented as a primary resistance determinant for penicillin and cefotaxime. When seven laboratory mutants were obtained through multiple selection steps of increasing the concentration of penicillin (4 mutants) and cefotaxime (3 mutants), each one contained a PB2x allele with up to five point mutations, including both known and new ones, in the transpeptidase domain (Figure 10.2). Out of these seven laboratory mutants, five contained Q552E substitution in their PB2x allele. The T550A substitution was the first amino acid substitution occurring in PB2x allele of one of the CTX in vitro mutants. Q552E and T550A are located in the vicinity of the K547SG motif and have been reported as major determinants of β-lactam resistance (Mouz et al. 1998; Mouz et al. 1999; Chesnel et al. 2002; Pernot et al. 2004) as they cause a decrease in the acylation efficiency of cefotaxime. G601V substitution, which has been shown to indirectly affect the active site of PB2x by introducing a bulkier side chain involved in topological alterations of the catalytic cleft (Maurer et al. 2008), was also present both in CTX (R6M3) and PG (1974M1). R384 substitution was found in two PG laboratory mutants (R6M1 and R6M2), but not in the CTX mutant. R384G substitution has been shown to lead to the instability of the SXN motif in the position 395-397 (Dessen et al. 2001). This instability results in a slight displacement of S395,
generating a more accessible, active site that may better accommodate alternative physiological substrates with branched stem peptides found in particular resistant clones (Dessen et al. 2001). The M289T substitution acquired by both R6M1 and R6M2 is located away from the catalytic cleft, and probably has only minor effects with respect to the active site of the protein, playing its role only in the background of other mutations (Maurer et al. 2008). Analyzing the genome of the seven laboratory mutants as well as known mutations in PBP2x revealed new mutations in PBP2x, including the Q281P, A369V, F388L, G408E, A507V, V518I/L, P522L, Y524C, M527T, P535L, V573L and V587L, each one which could be involved in resistance to β-lactams antibiotics (Figure 10.2). Independently introducing into sensitive R6 the PCR fragment containing PBP2x allele derived from the three laboratory CTX mutants produced transformants with a CTX MIC of 0.5μg/ml. When the PBP2x from PG laboratory mutants were introduced into wild-type R6, however, transformants with a PG MIC of 0.06μg/ml were obtained. In this work, selection in the laboratory demonstrated that PBP2x is the primary resistance determinant in the case of both PG and CTX because mutations of PBP2x occurred before the selection of other PBP mutations during the selection process.

We have not found any mutation in PBP2b of CTX laboratory mutants which is consistent with the fact that cefotaxime does not react with PBP2b (Hakenbeck et al. 1987). In four PG laboratory mutants selected in this study, each one was observed to contain a PBP2b allele with up to four new point mutations. Most of these mutations were located in the vicinity of the second and third active site motifs, S443SN and K615TG. The T451A and G435S substitutions were the only common mutations observed in three and two PG mutants, respectively (Figure 10.2). The A395V, D415E, T431D, L492F, Q633E and E665D substitutions have been found only in one mutant and could be involved in resistance. Introduction of both mutated PBP2x and PBP2b into sensitive strain resulted in transformant with a penicillin MIC of 0.125μg/ml to, which was 8 fold lower than the MIC of original mutants.
Figure 10.2 New mutations in PBP 2x, 2b and 1a found in our study. The transpeptidase domain is shown in black bar TP domain. The active site motifs are marked with triangle on top. All mutations in black are the ones already found in β-lactams laboratory mutants and new mutations identified in our study shown in red. (modified from (Hakenbeck et al. 2012)).

PBP1a may be considered as the most important PBP clinically, because mosaic PBP2x and PBP2b in the presence of an intact PBP1a allele provided only low level of
resistance, and β-lactam drug still have some efficacy (Zapun et al. 2008). Resistance mediated by PBP1a, can only be detected in the presence of a low affinity PBP2x and/or PBP2b, suggesting that there is an interaction between these PBP at some level (Hakenbeck et al. 2012). PBP1a mutations have not been observed in laboratory mutants (Hakenbeck et al. 2012). However, out of four PG mutants selected in our study, three contained nonfunctional PBP1a and the other mutant acquired G544R substitution which is in the vicinity of the third active site motif K557TG (Figure 10.2). Surprisingly, we did not found any increase in resistance level to PG following introduction of nonsense mutation into R6 with mutated PBP2x and PBP2b. But further experiment showed that PBP1a might have a role in PG resistance of laboratory mutant. Indeed, the reversion of the mutation in R6M1 to wild-type sequences resulted in a twofold decrease in resistance, suggesting that a putative PBP-unrelated genetic background is required for PBP1a to participate in resistance. In the case of laboratory CTX mutants, PBP1a was not mutated.

Compare to other PBPs, PBP2a has a relatively low affinity for β-lactam antibiotics especially to penicillin (Du Plessis et al. 2000; Zhao et al. 2000), suggesting that PBP1a mutations are therefore selected before mutations in PBP2a are contributing to resistance (Zhao 2000). However, an A463E substitution close to the SLN catalytic motif of PBP2a has been selected in the two CTX mutants (R6M1 and R6M2) and transformation experiment confirmed that this mutation results in two fold increase in CTX MIC when mutations in PBP2x are present. The absence of PBP2a due to premature transcription in cefotaxime laboratory mutant (Hakenbeck et al. 2012), and an altered PBP2a containing T411A mutation flanking the active site Ser410 was observed frequently in clinical isolates (Du Plessis et al. 2000; Smith and Klugman 2005; Carapito et al. 2006).

The T242K mutation located adjacent to the active site motif K239TG in the low molecular weight PBP3 was observed in R6M2 CTX mutant and its role in resistance confirmed by transformation experiments. Although the precise role of this mutation in resistance remains to be established, it is intriguing that a similar T242I change had previously been detected in S. pneumoniae laboratory mutants resistant to CTX (Krauss
and Hakenbeck 1997). A reduced amount of PBP3 due to mutation in the promoter region was also found in one laboratory mutant resistant to cefotaxime (Selakovitch-Chenu et al. 1993).

Using whole genome transformation strategy, we reconstructed penicillin resistant by transforming genomic DNAs derived from three penicillin resistant clinical isolates into sensitive wild-type cells. As might be expected, the genome sequence analysis of the resistant transformants revealed that all three transformants had multiple amino acid substitutions in PBPs 2x, 2b and 1a, while altered PBP2a has been found in two transformants, T3-55938 and T5-1983. The increase in penicillin (PG) resistance was mainly due to multiple changes in PBP alleles. Whole genome sequencing of clinical isolates transformants have shown that penicillin resistance in one of clinical isolate was fully explainable with mosaic \textit{php} genes while in two other clinical isolates these mosaic genes did not explain fully resistance. These data suggest that mechanisms other than PBPs could be implicated in penicillin resistance of clinical isolates.

10.4 The putative iron permease and penicillin resistance

Even though PBPs have been identified as the primary \(\beta\)-lactam resistant determinants, the introduction of all PBP mutations present in our PG laboratory mutants into penicillin-susceptible strain did not led to the resistance level found in the original laboratory mutant. This implies that other mutations are probably involved in resistance. The analysis of recurrent mutations in our panel of resistant strains, pinpointed a nonsense mutation in a putative iron permease that occurred early during the selection process, before any PBP mutations could be selected. In previously characterized laboratory-derived penicillin and cefotaxime-resistant mutants (Hakenbeck et al. 1994), PBP variants associated with resistance occurred late during the selection process (Krauss et al. 1996; Rogers et al. 2007), suggesting that the initial increase in resistance during the first steps of selection involves non-PBP mutations. We believed that the recurrent mutation in the iron premease could be part of this. We inactivated the gene encoding for the iron permease in a sensitive strain, but its inactivation did not lead directly to penicillin resistance. Nor did we find a direct role in resistance to penicillin by introducing the nonsense mutation of the iron permease in
a cell background with mutated PBPs. Intriguingly, the bactericidal activity of antibiotics like β-lactams has recently been linked to the iron-dependent accumulation of reactive oxygen species (ROS) (Kohanski et al. 2007). This study has argued that three main classes of bactericidal antibiotics, including β-lactams (penicillin, PG), fluoroquinolones (ciprofloxacin, CIP) and aminoglycosides (kanamycin, Kan), regardless of their primary targets kill bacteria by inducing alterations in iron homeostasis, ultimately leading to the accumulation of hydroxyl radicals through the Fenton reaction (Kohanski et al. 2007). Thus we sought to determine whether the acquisition of the nonsense mutation in the putative iron permease could translate into decreased accumulation of ROS following exposure to penicillin. We indeed found that penicillin stimulate a greater production of ROS in the presence of a functional version of the iron permease. We next tested whether this was a more general feature of bactericidal antibiotics and we found that ciprofloxacin and kanamycin also induced significantly more ROS in cells harboring an unaltered iron permease while the bacteriostatic antibiotics chloramphenicol and tetracycline, failed to induce ROS even in the presence of a WT version of the iron permease and only induced a slight accumulation of ROS that was not correlated to the functional status of the iron permease. These results led us to conclude that the time-dependent accumulation of ROS was a specific feature of bactericidal antibiotics. Even though the transformation of nonsense mutation of the iron permease under a background of mutated PBPs did not reveal a direct role in resistance to penicillin, our hypothesis is that its early inactivation could have provided increased protection against the accumulation of ROS during the selection of resistance by potentially decreasing the availability of free iron. Moreover, the R6M2 mutant further harbors a missense mutation in another putative iron uptake system (spr0934), which could potentiate the protective effect conferred by the spr1178 inactivation. The analysis of a panel of five penicillin non-susceptible clinical isolates failed to show similar nonsense mutations in spr1178, but this might in part be explained by the obvious growth defect associated with the acquisition of this mutation. It is relevant to point out that the exposure to sub-lethal concentrations of bactericidal antibiotics was shown to induce a decreased expression of iron uptake systems in *Pseudomonas aeruginosa* (Mikkelsen et al. 2010) and *S. pneumoniae*
(Rogers et al. 2007), so similar gene expression alterations could also potentially occur in clinical isolates to prevent the accumulation of ROS during the early steps of resistance selection, instead of more drastic events like nonsense mutation as observed in isolates selected in vitro.

10.5 The role of non-PBPs in cefotaxime and penicillin resistance

While mutations in PBPs contributed to CTX resistance in every mutant, introduction of altered PBPs alone into a susceptible strain by sequential transformation never led to the high level of CTX resistance of parental mutants. There is considerable evidence suggesting that non-PBP resistance mechanisms (Guenzi et al. 1994; Grebe and Hakenbeck 1996; Filipe and Tomasz 2000; Smith and Klugman 2001; Soualhine et al. 2005; Crisostomo et al. 2006; Dias et al. 2009; Tait-Kamradt et al. 2009) can be implicated in resistance to β-lactams. Whole genome sequencing of laboratory CTX mutants in this project highlighted novel resistance determinants.

One of the non-PBP mutations specifically selected in R6M1 was a stop mutation in spr1098, encoding for sortase A. The mutation found in spr1098, those in two other genes (spr1120 and spr1870), and the altered PBPs were sufficient to fully reconstruct resistance in the T3M1 obtained through whole genome transformation. We failed, however, to introduce the mutated spr1098 into *S. pneumoniae* with an altered PBPs background. Target sequencing of spr1098 in different transformants obtained following WGT of R6M1 revealed that this mutation was selected at the third (last) round of WGT, suggesting that probably bacteria needed the compensatory mutation to tolerate the burden of mutations in both PBPs and SrtA, which could explain our failure in introducing mutated spr1098 into *S. pneumoniae* with background of altered PBPs. Replacing the mutated spr1098 allele from T3M1 by a functional version of the gene caused a four-fold sensitization to CTX, which was consistent with our hypothesis, confirming the role of sortase A in CTX resistance. SrtA is a highly conserved transpeptidase responsible for the covalent attachment of LPXTG-motif-containing proteins to the cell wall (Clancy et al. 2010). The genome of *S. pneumoniae* R6 codes for more than ten proteins containing a LPXTG motif at their C-terminal region and
possibly the CTX resistance phenotype conferred by the non-sense mutation within SrtA is due to the miss-localization of one (or more) of these proteins. Interestingly, a decreased expression of sortase-encoding genes as well as several surface protein-encoding genes has been observed in vancomycin-resistant *Staphylococcus aureus* isolates (McAleese et al. 2006), suggesting a possibly more global role for sortases in response to cell wall-induced stresses. Additional work will be required to study the putative role of sortase in cefotaxime resistance. A comparative proteomic approach is suggested as a possible strategy to detect differences in surface proteins abundance following the introduction of sortase mutation into *S. pneumoniae* R6 wild-type.

The gene spr1333 coding for the peptidoglycan GlcNAc deacetylase, *pdgA* (Vollmer and Tomasz 2000) was mutated in two CTX mutants, R6M2 and R6M3. In *S. pneumoniae*, a significant proportion of the GlcNAc residues within the cell wall are N-deacetylated and the inactivation of *pdgA* was shown to produce cells expressing fully N-acetylated glycans that are more sensitive to lysozyme (Vollmer and Tomasz 2000) in addition to be less virulent in an intra-peritoneal mouse model (Vollmer and Tomasz 2000). While the precise role of PdgA in β-lactam resistance remains to be clarified, it is intriguing that alterations in the level of O-acetylation of peptidoglycan muropeptides influence the level of resistance to penicillin in *S. pneumoniae* (Crisostomo et al. 2006). A *pdgA* point mutation implicated in resistance to β-lactams has also been described upon transformation of *S. pneumoniae* R6 with gDNA derived from a penicillin-resistant clinical isolate (Tait-Kamradt et al. 2009). The role of spr1333 mutation in CTX resistance in the background of altered PBPs was confirmed by transformation experiment. The mutated spr1333 with altered PBPs led to the increase in CTX resistance to same level as that of the parental one, i.e. the R6M2.

The mutations in *strA* and *pdgA* could respectively explain CTX resistance of R6M1 and R6M2 that was not due to mutations within PBPs. While the CTX MIC of R6M3 was 8.0μg/ml, higher than the two other mutants, the contribution of PBP2x, the sole mutated PBP in this mutant, in CTX resistance was up to 0.5μg/ml. In R6M3, mutated spr1333 with altered PBPs increased the resistance level but not as high as in the original mutant. Since R6M3 was generated in a step by step manner with distinct
CTX increments, we expected to find more than one non-PBP genes involved in CTX resistance. By looking at the order of appearance of mutations found by whole genome sequencing in the 1M3 at 1.0μg/ml, 2M3 at 2.0μg/ml, we selected some candidates which seemed to be critical for conferring higher resistance against CTX. Our transformation experiment revealed that the mutations in spr1704 (an ABC transporter), spr1333 (PdgA) and spr0981 (CpoA) were responsible for differences in the resistance level to CTX between transformants.

spr1704, amiE, is a part of the operon ami which is an oligopeptide transport system, composed of five subunits: an oligopeptide binding protein, amiA; two transmembrane proteins, amiC and amiD; and two ATP binding proteins, amiE and amiF, which provide the energy for oligopeptide transport (Gardan et al. 2009). Recently, one of the oligopeptide transporter systems (Ami) of S. thermophilus was reported to be involved in the import of several pheromones and triggering competence (Gardan et al. 2009). The Ami oligopeptide transporter system of S. pneumoniae is also involved in the colonization of the nasopharynx (Kerr et al. 2004). In a recent study, they suggested that the oligopeptide transporter may function as major transporter for nutrient uptake in S. pneumoniae (Choi et al. 2010).

While the role in resistance for altered alleles of cpoA was thought to be restricted to penicillins (Grebe et al. 1997; Hakenbeck et al. 1999), point mutation in CpoA from R6M3 clearly conferred a four-fold increase in CTX resistance. Prokaryotic CpoA homologues are involved in teichoic acid or LPS biosynthesis and the S. pneumoniae CpoA was shown to act as a lipid glycosyltransferase by transferring a galactose moiety to monoglucosyl-diacylglycerol (α-MGlCDAG) to produce galactosyl-glucosyl-diacylglycerol (GalGlcDAG), the main glycolipid in S. pneumoniae (Edman et al. 2003). It is thus possible that altered cpoA alleles may result in a modified synthesis of the polymer, thereby altering indirectly the property of the cell envelope and counteracting the activity of cell wall antibiotics.

Study of penicillin resistant clinical isolates in addition to confirming the role of PBPs in resistance, has highlighted spr1239 (an alpha-amylase) as a new marker of
clinical penicillin resistance. It is part of the sixteen *S. pneumoniae* genes encoding proteins putatively involved in the metabolism of α-glucans (Abbott et al. 2010), of which six (including spr1239) have been identified to be necessary for the full virulence of the bacterium by signature-tagged mutagenesis (Hava and Camilli 2002). Given that *S. pneumoniae* also have numerous extracellular enzyme systems that allow the metabolism of a large number of sugars that can flow directly into the glycolytic pathway (Tettelin et al. 2001), the precise role in PG resistance for the non-sense mutation in the spr1239 alpha-amylase will require further investigations.

There are several mutated genes in the mutants and in the transformant for which we did not found any contribution to resistance. For example, respectively 8 and 13 mutations in the CTX R6M1 and R6M3 mutants could not be linked to resistance. Similarly, 2 and 4 mutations in the transformant T3M1 and T3M2 could not be linked to resistance. These types of mutations could be simply the error of replication or could be implicated in the fitness cost or served as compensatory mutations. These mutations were more abundant in the mutants compared with the transformants. Reconstruction of resistance using whole genome transformation is likely to decrease the number mutations due to other factors such as replication’s error.

### 10.6 DNA transformation at the genome scale level

Genome sequencing of three transformants obtained following transformation of genomic DNAs of three penicillin resistant clinical isolates into *S. pneumoniae* R6, has allowed to further study DNA transformation at the genome scale level. Analysis of sequencing results showed that the three transformants, T2-18209, T5-1983 and T3-55938 had replaced respectively 16.2 kb, 82.7 kb and 137.2 kb of their genomes with 3, 10 and 23 multiple-gene recombinant sequence segment (RSSs) of the respective parental clinical isolates, documenting the extent of DNA transformation between strains. The length of these 36 RSSs ranged from 1.4 to 19.4 kb, with an average size of 6.5 kb. These lengths are similar to those reported from analysis of naturally occurring recombination tracts observed in *Neisseria meningitides* (5.1 kb) at specific loci (Linz et al. 2000) and in *H. influenza* (8.1 kb) (Mell et al. 2011), but larger than the 2kb
found in a recent study with high-level penicillin resistant *S. mitis* B6 DNA (Sauerbier et al. 2012).
Conclusion

Our belief was that genomics would accelerate our understanding of antibiotic resistance and could lead to the discovery of new biomarkers. We have used successfully NGS for determining mechanisms of penicillin and cefotaxime resistance in *S. pneumoniae*. The work reported in this thesis helped to confirm some known mechanism of resistances, to highlight some new genes and to improve knowledge related to β-lactam resistance in *S. pneumoniae*. We demonstrated the value of whole genome transformation as a useful strategy for reconstructing resistance both in vitro isolates and clinical strains.

Conclusion of this work is that mutations in PBPs are not sufficient to achieve high level resistance to penicillin in laboratory and clinical isolates. These mutations may help the selection of other mutations such as those within a putative iron transporter. The next genome sequencing data further revealed other mutations that were acquired by at least one mutant, proposing that some of these, or a combination of mutations, could be associated with penicillin resistance along with mutations in PBPs. Our works on cefotaxime resistance confirmed the role of PBPs in resistance in in-vitro mutants and showed that mutations in spr1333, spr0981, spr1704 and spr1098 coding respectively for a peptidoglycan GlcNAc deacetylase, a glycosyltransferase, an ABC transporter, and a sortase were implicated in resistance to cefotaxime.

Whole genome sequencing of β-lactams mutants in this thesis has pinpointed some new markers involved in resistance. It would be interesting to do further functional work, to monitor SNPs-mediated changes in expression through RNAs , proteomic and metabolomics approaches in order to complement genomic studies of antimicrobial resistance and to determine the biochemical and cellular basis by which these new markers confer resistance in *S. pneumoniae*. 
References


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