Streptococcus pneumoniae: molecular epidemiological aspects and the identification of virulence factors

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Streptococcus pneumoniae: moleculair epidemiologische aspecten en de identificatie van virulentie factoren

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General introduction

Introduction

Streptococcus pneumoniae (the pneumococcus) is a lancet-shaped gram-positive bacterium, which grows in pairs or short chains (Fig. 1). The pneumococcus is a human pathogen that

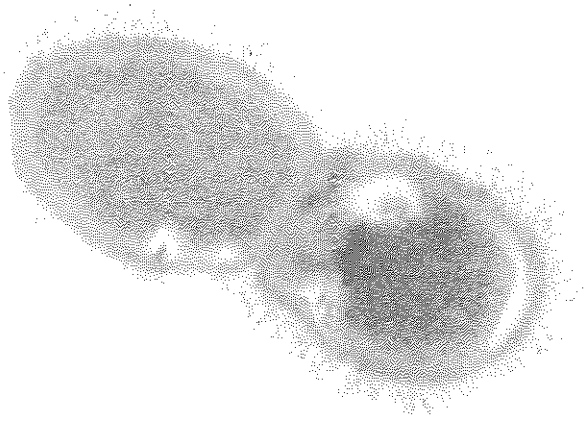

Fig. 1. Electron micrograph of *S. pneumoniae*.

forms a leading cause of invasive diseases including pneumonia, bacteremia and meningitis. *S. pneumoniae* is also the worlds major cause of otitis media and sinusitis. These are less serious non-invasive diseases, which, however, have a significant impact on health-care costs because of their high prevalence. The incidence of pneumococcal disease is highest in young children, elderly and immunocompromised individuals. Invasive pneumococcal infections are associated with significant mortality, particularly in developing countries where many children under the age of five years die each year from pneumococcal pneumonia.

History

S. pneumoniae was first isolated in 1881 by George M. Sternberg (107) and Louis Pasteur (85). Both scientists have isolated the pneumococcus from carriers. The association of the pneumococcus with pneumonia and with infections of other body sites was elucidated in the

same decade. Studies on the pneumococcus have contributed to the understanding of many fundamental biological principles (for review see references 5, 6, 116). Gram has developed a staining procedure that is still in everyday use in bacteriological laboratories (44). The pneumococcus was one of the first pathogenic bacteria observed by the Gram-stain. The pneumococcus has also played a role in the discovery of the protective value of antiserum. The Klemperers have shown that serum from rabbits injected with heat-killed pneumococci contained factors that conferred protection against reinfection with the same strain. More importantly, rabbits were protected against infection by infusion of serum from a previously immunized animal (66). These studies have resulted in the development and successful application of serum therapy. Issaeff has shown that the protective serum was not directly bactericidal but did promote uptake of pneumococci by phagocytic cells of the immune system (61). Neufeld has described the Quellung reaction or the macroscopic agglutination and microscopically visible swelling of the external capsule upon addition of specific antiserum to a suspension of pneumococci (81). This technique, designated serotyping, has become an important tool for bacterial typing. The antigenicity of polysaccharides has first been demonstrated with capsular polysaccharides from the pneumococcus. Furthermore, anti-polysaccharide antibodies have shown to provide type-specific protection against pneumococcal challenge (53, 54). This has opened the way for the development of vaccines of pneumococcal capsular polysaccharides. In 1929, Fleming has discovered the antibacterial properties of a fungus-derived substance (41). The first patient that showed clinical benefit from this substance, which was called penicillin, was suffering from pneumococcal conjuctivitis. Since then, penicillin has been the antibiotic of choice to treat pneumococcal infections. The introduction of penicillin has dramatically reduced the mortality associated with invasive pneumococcal infections. The most important contribution to biology arising from studies on the pneumococcus, is the discovery of the principle of capsular transformation. Griffith has discovered that mice injected with living rough (non-encapsulated) pneumococci and mice injected with heat-killed smooth (encapsulated) pneumococci survived. However, mice injected with both living rough pneumococci and heat-killed smooth pneumococci died. He was able to isolate live smooth pneumococci from blood (46). At that time, the significance of this transforming principle was not understood. In 1944, Avery and coworkers have shown that "acidic compounds" (DNA) constituted the genetic material that as a result of transformation was responsible for the phenotypic changes (9).

Epidemiology

The serotyping method described by Neufeld is used for epidemiological studies. To date, as many as 90 different serotypes of *S. pneumoniae* have been identified on the basis of antigenic differences in their capsular polysaccharides (55). Certain serotypes are known to be more virulent than others. Most of the infections are caused by a restricted number of serogroups and serotypes. In addition, the distribution of serotypes varies in time and in different age groups, populations and geographic areas (26, 82, 100, 102). The serogroups causing most invasive diseases in adults are 1, 3, 4, 7, 8, 9, 12 and 14, while the most frequently occurring invasive serogroups in children are 4, 6, 9, 14, 18, 19 and 23. The latter serogroups are most prevalent in the United States (78% of the isolates), Finland (84% of the isolates), and Australia (78% of the isolates). However, in Rwanda (33% of the isolates) and Papua New Guinea (31% of the isolates) these serogroups play a minor role in pediatric invasive disease (23).

Until the 1960s, pneumococci were considered uniformly susceptible to penicillin. However, in the last 30 years, increasing numbers of penicillin-resistant pneumococcal clinical isolates with rising MIC values are isolated all over the world (47). Penicillin-resistance is mostly associated with "pediatric" serogroups 6, 9, 14, 19 and 23 (47). Many penicillin-resistant isolates are multiple antibiotic-resistant and isolates with resistance to tetracycline, chloramphenicol, rifampicin and erythromycin are increasingly encountered (4, 56). The emergence of high-level resistance to penicillin, particularly in combination with other resistance determinants, is a serious threat for the effectiveness of current treatment strategies. Serious pneumococcal infections caused by multiple antibiotic-resistant pneumococci are usually treated with third generation cephalosporins and/or vancomycin. However, high-level cephalosporin resistance is increasingly present within the pneumococcal population (40). Recently, clinical isolates with tolerance to vancomycin, the antibiotic of last resort, were isolated (72, 84). Antibiotic tolerance, which is characterized by the ability of bacteria to survive but not grow in the presence of antibiotics, is of clinical significance, since it has been shown that the inability to eradicate tolerant bacteria leads to failure of antibiotic therapy. Furthermore, tolerance is thought to promote the development of antibiotic resistance (83).

In addition to serotyping and the determination of antibiotic resistance, DNA fingerprinting methods have been used for epidemiological surveys. The last methods are required to provide greater discrimination and complementary information (48). DNA typing methods

such as pulsed field gel electrophoresis, BOX PCR fingerprinting and restriction fragment end labeling provide genomic information that is suitable to identify the genetic relatedness between strains (58). Fingerprinting of the penicillin-binding protein genes adds further information to the analysis of penicillin-nonsusceptible strains. The use of a combination of typing methods has led to the knowledge that this naturally transformable species undergoes frequent horizontal gene transfer, which has resulted in a freely recombining population structure with epidemic spread of successful clones (49). The worldwide increase in antibiotic resistance and the clinical implications of penicillin resistance has focused most studies on the molecular epidemiological characteristics of penicillin-resistant isolates. Soares et al. have reported the epidemic spread of a multi-resistant serogroup 6 pneumococcal clone in Iceland, and have suggested that this single clone has been imported from Spain to Iceland (106). Munoz and colleagues have documented the intercontinental spread of a multi-resistant clone of serotype 23F from Spain to the United States (78). Gasc et al. have described the relatedness between French serogroup 9 isolates and earlier Spanish isolates indicating the spread of this penicillin-resistant clone form Spain to France (43). The serotype 23F and 9V clones have been identified in many countries all over the world (56, 57, 104). In addition, novel penicillin-resistant and multi-resistant clones tend to spread in an epidemic manner within other countries (26, 39, 94, 105, 124).

Mechanisms of antibiotic resistance

Penicillin resistance has emerged in *S. pneumoniae* through the development of altered penicillin-binding proteins (PBPs) with decreased affinity for penicillin and other β-lactam antibiotics. The pneumococcus possesses five high molecular weight PBPs (1a, 1b, 2a, 2b, 2x) and the low molecular weight PBP3. Altered PBPs are encoded by mosaic genes that have emerged by recombination events between *pbp* genes of pneumococci and their homologues in closely related streptococcal species. Some mosaic *pbp* genes are composed of a single block, and others are more complex being the result of genetic recombination with several donors (36). Furthermore, there is an enormous diversity of mosaic *pbp* genes, suggesting that resistance to penicillin has arisen on many occasions around the world. Resistance to third generation cephalosporins is also based on changes in the penicillin-binding proteins (27).

In contrast, resistance to non-β-lactam antibiotics is based on other mechanisms. Erythromycin resistance is often the result of modification of the ribosome through methylation of 23S rRNA. This methylation is the result of methylase activity, encoded by *ermAM*. Resistance to erythromycin can also be the result of efflux of the antibiotic from the cell by an efflux pump encoded by *mefE* (122). The production of chloramphenicol acetyltransferase, an enzyme capable of catalyzing the conversion of chloramphenicol to its nonfunctional derivates, leads to chloramphenicol resistance. Chloramphenicol acetyltransferase is encoded by *cat* identical to *cat* from a *Staphylococcus aureus* plasmid (122). Resistance to tetracycline occurs through ribosomal protection encoded by the genes *tetM* and *tetO*, although the precise resistance mechanism remains unclear (122). Trimethoprim resistance is mediated through a single amino acid substitution in the chromosomal dihydrofolate reductase, which is thought to disrupt the bond with trimethoprim without affecting the action of dihydrofolate reductase (122). Sulphonamide resistance results from repetitions of one or two amino acids in the chromosomal dihydropteroate synthase (122). Rifampin resistance in *S. pneumoniae* has been shown to be due to mutations within the target of the antibiotic, the β-subunit of RNA polymerase encoded by *rpoB* (37). Fluoroquinolone resistance is mediated by changes in the target of fluoroquinolones i.e. DNA gyrase (*gyrA*) and/or topoisomerases (*parC, parE*), and/or decreased intracellular accumulation as a result of enhanced efflux by the efflux pump PmrA (89). Recently, the mechanism of vancomycin tolerance in *S. pneumoniae* has been reported to be the result of loss of function of the VncS histidine kinase of a two-component sensor-regulator system. Two-component systems monitor environmental parameters through a sensor histidine/phosphatase, which phosphorylates/dephosphorylates the response regulator that in turn mediates changes in gene expression. Novak and colleagues have suggested that signal transduction is critical for the bactericidal activity of vancomycin (84).

The pathogenesis of pneumococcal infections

The human nasopharynx forms the normal ecological niche of *S. pneumoniae*. The entire human population is colonized once or several times during their life by the pneumococcus. At one time, up to 60% of the individuals can be carrier (7). The prevalence of carriage is especially high in young children, elderly and immunocompromised persons, and in crowded places such as day care centers, hospitals, military academies, jails and nursing homes.

During carriage, more than one serotype can co-exist (8). Nasopharyngeal carriage of particular pneumococcal capsular types is often accompanied by the development of protection against infection by these types. Most infections do not occur after prolonged carriage but follow the acquisition of recently acquired strains (45). Pneumococcal infections start with the adherence and colonization of the epithelial cells of the nasopharynx. From there, pneumococci can spread locally, either into the Eustachian tube and into the middle ear cavity to produce otitis media, or into the alveoli of the lower respiratory tract to cause pneumonia. In some cases, pneumococci are able to enter the bloodstream and cause bacteremia or cross the blood-brain barrier and cause meningitis.

Virulence factors

The pneumococcus synthesizes several factors that play a role in the pathogenesis of infections (Fig. 2, Table 1). Most of these factors are surface-associated.

Capsule. The polysaccharide capsule has since long been recognized as the most important virulence factor. Experimental proof has been provided using transposon mutagenesis. Specific interruption of capsule production results in an increase of the lethal dose by a million fold (115). Studies with strains differing only in their capsular type have shown that the capsule has a major effect on virulence. However, the genetic background is also important, indicating that other components also contribute to pneumococcal virulence (62). The capsule provides resistance to phagocytosis (21), and thereby promotes the escape of pneumococci from the immune system of the host.

Cell wall and cell wall polysaccharides. The cell wall is mainly composed of peptidoglycan: glycan chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues, cross-linked to each other through peptide side-chains. The peptidoglycan layer anchors the capsular polysaccharides and cell wall polysaccharides. The cell wall polysaccharides consist of teichoic acids and phosphorylcholine (2). The latter structure forms an anchor for at least eight choline-binding proteins (96). It is also part of platelet-activating factor (PAF), and is therefore suggested to be important in the attachment to cytokine-activated human cells via the PAF receptor by structural mimicry (31).

The cell wall and cell wall polysaccharides induce inflammation similar to that observed during pneumococcal infection (110-112). Inflammation is caused by the activation of the

alternative complement pathway. During complement activation, the anaphylatoxins C3a and C5a are produced, which results in the enhancement of vascular permeability, induction of mast cell degranulation and chemoattraction of polymorphonuclear leucocytes to the inflammation site (2).

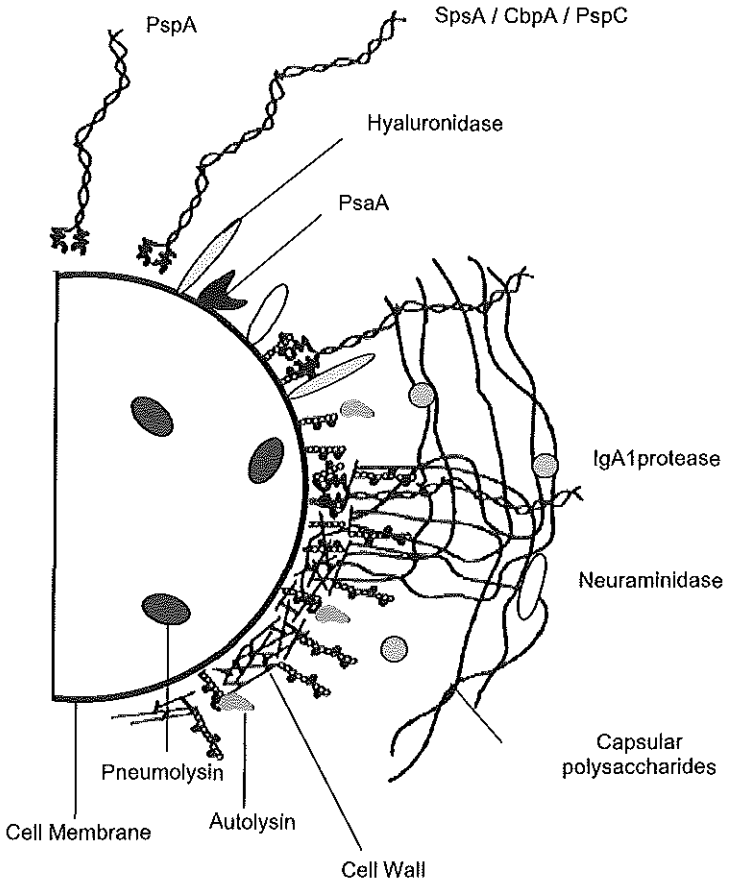

Fig. 2. Hypothetical representation of a pneumococcus. Adapted from Briles et al. (18).

Pneumolysin. Pneumolysin is one of the best characterized pneumococcal virulence proteins (for review see reference 76). This protein is produced by virtually all clinical isolates. Pneumolysin is an intracellular hemolysin and member of the family of thiol-activated cytolysins. It has a range of biological activities. Pneumolysin is lytic for all eukaryotic cells that have cholesterol in their membrane. The protein binds to membrane cholesterol and inserts into the lipid bilayer, followed by lateral diffusion and assembly of a transmembrane

pore structure. Pneumolysin is able to injure a range of eukaryotic cells and is presumed to account for much of the histopathology of pneumococcal infections (76). In addition, pneumolysin has a range of physiological effects on cells at sublytic concentrations, which can lead to inhibition of certain cellular functions such as bacterial killing by polymorphonuclear leucocytes and monocytes (79), and to stimulation of other pathways such as cytokine production, phospholipase activity (97) and activation of the classical complement pathway by binding to the Fc portion of antibody (77, 88). Furthermore, pneumolysin interferes with nonspecific host defense mechanisms such as slowing the cilial beat of human nasal epithelium and inhibiting the function of ciliated ependymal cells (76). Studies with mutants in which the pneumolysin gene has been disrupted, have demonstrated that pneumolysin clearly plays a role in the pathogenesis of pulmonary and systemic infections, although its role in meningitis is presumed to be limited (16).

Autolysin. Autolysin (*N*-acetylmuramyl-L-alanine amidase; LytA) is a cell wall degrading enzyme, which is anchored to the choline residues in the cell wall. The enzyme is normally inactive but under conditions when cell wall biosynthesis stops, such as nutrient starvation or penicillin treatment, the enzyme is activated and causes autolysis of the bacterial cell (75). Autolysis contributes to virulence, although indirectly, by releasing degradation products of the cell wall, which are highly inflammatory, and toxic cytoplasmic components such as pneumolysin (67).

Neuraminidase. Neuraminidase is an enzyme which cleaves *N*-acetylneuraminic acid from mucin, glycolipids, glycoproteins, and oligosaccharides on host cell surfaces (101). It has been proposed that neuraminidase is able to enhance colonization by decreasing the viscosity of mucus or by exposing cell surface receptors (3). All clinical isolates examined to date produce neuraminidase. The pneumococcus has at least two neuraminidases, NanA and NanB (24). The specific activity of NanA is much greater than NanB and NanB has a significant lower pH optimum (12). Examination of the partial *S. pneumoniae* genome sequence indicated the presence of a third neuraminidase exhibiting approximately 50% amino acid sequence identity to NanB, NanC (14).

Hyaluronidase. Hyaluronidase depolymerizes hyaluronic acid. The degradation of this important component of connective tissues and extracellular matrixes has been postulated to contribute to invasion. In addition, this enzyme may contribute to virulence or survival of the

pneumococcus in the host by scavenging potential carbon sources such as glucose and glucoronic acid (13). Virtually all clinical isolates produce hyaluronidase (86). Mutagenesis of the hyaluronidase gene has no significant impact on virulence in a mouse intraperitoneal challenge model (14).

Table 1. Characteristics of the major virulence factors of *S. pneumoniae.* Adapted from Alonso DeVelasco et al. (2).

Virulence factor	Proposed mechanism of virulence*	References
Capsule	Resistance to phagocytosis	(21)
	Lack of immunity of some capsular types	(59, 60)
Cell wall and cell wall polysaccharides	Inflammatory effects	(110-112)
	Mediator of attachment to host cells	(31)
Pneumolysin	Cytolytic at high concentrations	(76)
	Cytotoxic at lower concentrations	(76)
	Stimulation of cytokine production	(76)
	Activation of classical complement pathway	(77, 88)
Autolysin	Release of pneumolysin and cell wall components	(67)
Neuraminidase	Decrease of the viscosity of mucus or exposure of cell surface receptors ?	(3, 101)
Hyaluronidase	Degradation of connective tissue	(13)
IgA1 protease	Inhibition of mucosal defense mechanisms ?	(63)
PsaA	Cellular adhesion	(15)
	Zinc and manganese transport	(34)
	Trigger for autolysin expression	(83)
PspA	Inhibition of complement activation	(109)
	Involvement in iron uptake ?	(50)
CbpA	Cellular adhesion	(96)
	Cellular invasion	(95)
	Reduction of bacterial clearance	(51)

*The mechanisms marked "?" have been suggested but not demonstrated. Most of the mechanisms listed are observed only in vitro, their significance for virulence remains unknown.

Immunoglobulin A1 protease. Immunoglobulin A1 (IgA1) protease is a metalloprotease that specifically cleaves the heavy chain of human IgA1 (93, 114). IgA1 is the predominant class of immunoglobulins on mucosal membranes. Because of the specificity for human IgA1, no relevant animal models are available to test the biological function of IgA1 proteases. However, several lines of evidence suggest that IgA1 protease activity enables pneumococci to evade the local mucosal defense system and is an important colonization factor (63). Almost all clinical isolates show IgA1 protease activity (69).

Pneumococcal surface adhesin A. Pneumococcal surface adhesin A (PsaA) is a 37 kDa protein present on the surface of the bacterium and expressed by all pneumococcal strains studied so far (98, 99). *psaA* is part of the *psa* locus, which encodes an ATP-binding cassette type manganese permease complex (34). The protein is essential for virulence as judged by intranasal and intraperitoneal challenge of mice (15). Mutagenesis of *psaA* results in defective adhesion (15), reduced transformation frequency (34), and penicillin tolerance (83). The latter effect is explained by the involvement of PsaA in triggering autolysin (83).

Pneumococcal surface protein A. Pneumococcal surface protein A (PspA) is, like LytA, a choline-binding protein (125). PspA is found on all pneumococci and is required for full virulence. Mutants lacking PspA are cleared more rapidly from the blood of infected mice than the parent strains (74). Recently, it has been shown that heat-killed pneumococci lacking PspA fix more complement than heat-killed pneumococci possessing PspA (18, 19). Tu and colleagues have examined the influence of PspA on complement activation in vivo (109). These investigators have demonstrated that PspA inhibits complement activation. More C3b is detectable on PspA-deficient pneumococci than on PspA wild-type pneumococci, suggesting that PspA interferes with deposition of C3b on pneumococci. PspA is presumed to exert its virulence function by blocking recruitment of the alternative complement pathway, thereby reducing the effectiveness of complement receptor-mediated pathways of clearance (109). Recently, Hammerschmidt and colleagues have postulated that PspA might function as a lactoferrin-binding protein, suggesting the involvement of PspA in iron uptake, and thus its contribution to the pneumococcal growth under iron-limited conditions, i.e. the human host (50).

Choline binding protein A. Choline-binding protein A (CbpA) has been characterized as an adhesin and a determinant of virulence. CbpA-negative mutants have reduced adherence to

human epithelial and endothelial cells, and reduced virulence in an infant rat model of nasopharyngeal carriage (96). In addition, CbpA is involved in invasion through microvascular endothelial cells (95). The same protein is also designated *S. pneumoniae* secretory immunoglobulin A-binding protein (SpsA), and has been shown to bind human secretory IgA and secretory component via a hexapeptide motif (51, 52). Hammerschmidt and colleagues have hypothesized that SpsA might contribute to virulence by the reduction of bacterial clearance. Finally, the protein is designated pneumococcal surface protein C (PspC) by Brooks-Walter et al., who have described that PspC is homologous to PspA, elicits cross-reactive antibodies to PspA, and provides protection to pneumococcal bacteremia in mice (20).

Phase variation

S. pneumoniae undergoes spontaneous, reversible variation (phase variation), which is apparent as differences in colony opacity on transparent agar surfaces (118). These differences in colony morphology appear to be relevant to the pathogenesis of pneumococcal infections. Only transparent variants are able to establish dense and stable colonization of the mucosal surface of the nasopharynx in an animal model for pneumococcal carriage, whereas isogenic opaque variants are deficient in colonization (118). This correlates with the enhanced binding of transparent pneumococci to buccal epithelial cells and their glycoconjugate receptors when compared to opaque pneumococci (31). Similarly, the adherence of transparent pneumococci is enhanced to cytokine-stimulated human type II lung cells, and human vascular endothelial cells, as well as the receptors (*N*-acetyl-D-glucosamine and PAF receptor) that appear on these cells after cytokine stimulation (31). On the other hand, the opaque variant is more virulent in a mouse model for systemic infection (65). The increased virulence of the opaque variant in an in vivo model for sepsis correlates with decreased opsonophagocytic killing of opaque pneumococci in an in vitro phagocytosis assay (64). Finally, transparent variants have an increased capacity to cross the blood-brain barrier (95). The higher binding affinity of transparent pneumococci to the PAF receptor on microvascular endothelial cells is presumed to result in increased transcytosis of bacteria across these cells.

The relationship between the expression of several previously identified cell surface components and phase variation has been investigated. The opaque phenotype of the pneumococcus is associated with larger amounts of capsular polysaccharide compared to the

transparent phenotype. This results in decreased opsonophagocytosis and a higher virulence in an mouse model of systemic infections of the opaque variant (64, 65). In contrast, the transparent pneumococcal phenotype as compared to the opaque variant produces increased amounts of cell wall polysaccharides, i.e. phosphorylcholine, which is thought to be responsible for the differences in adhesion between the two phenotypes (65, 117). The expression of four virulence proteins has been examined in the pneumococcal phase variants. The expression of pneumolysin is similar in both variants (65). In contrast, the choline-binding proteins LytA, PspA and CbpA have demonstrated differential expression. The opaque variants have decreased expression of autolysin and consequently undergo spontaneous lysis more slowly than transparent variants (119). PspA is expressed in greater amounts in the opaque variant than in transparent variants (65). CbpA is differentially expressed in pneumococcal phenotypic variants such that transparent variants express increased amounts (96). The ability of transparent variants to transcytose through microvascular endothelial cells, one of the cells lining the blood-brain barrier, was demonstrated to be dependent on CbpA expression (95).

Natural transformation

Genetic plasticity plays a central role in the biology of *S. pneumoniae*. This naturally transformable microorganism has the ability to adapt to environmental changes by horizontal transfer of genetic material. Horizontal gene transfer enables the bacterium to evolve rapidly by the acquisition of novel determinants and has resulted, under the selective pressure from host defenses and because of antibiotic treatment, in a great variability of several virulence factors and resistance determinants (for review see references 25, 36, 70).

One of the most elegant examples of horizontal gene transfer are the mosaic structures in the penicillin resistance genes. Low-affinity PBPs have arisen from interspecies recombination i.e., transformation and homologous recombination of DNA from viridans streptococci. *pbp* genes of resistant isolates are composed of mosaic blocks that are either similar or identical to *pbp* genes from sensitive isolates and blocks that are similar or identical to genes from *S. mitis* and *S. oralis* (91).

The pneumococcal polysaccharide capsule also exhibits a high-level of diversity. As many as 90 different capsular types have been described (55). In addition, there are still capsular types that can not be determined by the 90 distinct capsular antisera (121). Several studies indicate frequent horizontal transfer of capsular genes in vivo (10, 28, 29, 56, 80). The molecular

organization of the genes responsible for capsule biosynthesis has revealed a cassette-type organization with conserved regions flanking a cluster of genes that confer capsule specificity (42). Recombination events are therefore feasible to occur between the conserved flanking regions (29).

Mosaic structures have also been identified in surface-exposed and secreted virulence proteins, such as autolysin, neuraminidase A, IgA1 protease and PspC. PspC, also designated as SpsA, CbpA and PbcA, has been described as a chimeric protein that has acquired domains from both intraspecies genetic exchanges with *pspA* of other pneumococcal strains and interspecies genetic exchanges with DNA from group B streptococci (20). Interspecies genetic exchanges with strains of *S. mitis* have been described for the gene encoding IgA1 protease (92) and the neuraminidase gene (36) of *S. pneumoniae*. Although the autolysin encoding gene *lytA* displays limited genetic variation, recombination between host *lytA* and genes encoding cell wall lytic enzymes of pneumococcal bacteriophages (120) or *lytA* from pathogenic viridans streptococci is highly suggestive (36).

Host defense

Host protection against pneumococcal infection is mainly mediated by phagocytosis (123). Pneumococci are cleared from the lung and the blood by phagocytosis and subsequent intracellular killing by neutrophils, alveolar macrophages and phagocytic cells (22). This mechanism depends on the presence of opsonins, i.e. immunoglobulins and complement. Pneumococci are able to activate the alternative complement pathway. Since the alternative complement pathway can be activated in the absence of acquired specific antibodies, it is expected to be important for the naive host. Pneumococci are also activators of the classical complement pathway. In contrast, this requires the participation of specific antibodies. Therefore, the classical pathway is expected to play its protective role in the immune host. Activation of both the classical and alternative complement pathways results in the fixation of C3b to the pneumococcal surface, following cleavage of C3b to iC3b and C3d (123). Differences in cleavage patterns among pneumococcal serotypes help to explain serotype specific differences in susceptibility to phagocytosis and in capsular antigenicity. C3d is recognized by receptors on the B lymphocyte, but not by those on the phagocyte. In contrast, iC3b readily serves as a ligand for complement receptor type 3 on the neutrophil, thereby enhancing phagocytosis and clearance. Pneumococcal surfaces that halt C3 cleavage at iC3b will not generate C3d. For example, capsular types 3 and 4 are highly resistant to

phagocytosis and yet potent immunogens. C3d is the most predominant C3 fragment found on these capsular types. In contrast, capsular types 6A and 14 display only the cleavage fragment iC3b, which protects them from interaction with B lymphocytes but allows them to be easily phagocytosed (59, 60). *S. pneumoniae* is not susceptible to the lytic action of complement (123).

Pneumococcal vaccines

The current vaccine consists of the 23 most prevalent pneumococcal serotypes. The use of this 23-valent vaccine has eliminated only a small fraction of the morbidity and mortality associated with this organism. The first and most important explanation is the underutilization of this vaccine (38). A second explanation is the relatively low (±60%) effectiveness in the elderly against bacteremic infections caused by the 23 capsular types included in the vaccine (103). In addition, vaccination does not elicit adequate antibody responses in children less than 2 years of age who are an important risk group (35). Finally, antibodies to certain polysaccharide antigens, especially to capsular type 6B, are not protective (1). The current pneumococcal vaccine strategies focus on the use of conjugates, in which a limited number of different capsular polysaccharides are linked to a carrier protein. The conjugated proteins are able to switch the immune response against the polysaccharides present in the vaccine from T-cell independent to T-cell dependent. This will result in an increased antibody response against the polysaccharides and will induce a memory response. Conjugate vaccines are more immunogenic in young children than polysaccharide vaccines (23, 33). However, the number of different capsular polysaccharide types that can be included in these vaccines is restricted (90). In this respect, the ability of pneumococci to change capsular type is of obvious importance in relation to the development of pneumococcal conjugate vaccines. The apparent ease by which capsular types change through recombination suggests that pneumococcal conjugate vaccines may in long-term be less effective to prevent disease. Indeed, current studies show that vaccination results in a shift in serotype distribution towards those pneumococcal capsular polysaccharides that are not present in the vaccines (32, 71). The selective pressure for this shift is presumed to be formed by the human immune response that, after vaccination, selects for isolates with non-vaccine capsular types and for recombination of capsular genes encoding the biosynthesis of capsular polysaccharides that are not present in the vaccine. A major threat is the acquisition of non-

vaccine capsular types by antibiotic-resistant pneumococci. Recently, the Kaiser Permanente Vaccine Study Center Group has reported the results of a large double blind trial of a novel heptavalent conjugate vaccine in healthy children. They have demonstrated that the vaccine was highly effective in preventing invasive disease in young children. They also did not find any evidence for an increase of invasive disease by non-vaccine serotypes (17). However, the effectiveness in preventing otitis media was not as good as the effectiveness in preventing invasive disease. Molecular epidemiological studies will need to be performed in order to investigate the long-term effects of large-scale vaccination.

Over the last few years, much attention has been focused on the role of pneumococcal proteins in pathogenesis and protection. Proteins that are involved in the pathogenesis of infections by *S. pneumoniae* are considered interesting components for future conjugate and protein vaccines. Such proteins should ideally provide protection against colonization and infection with *S. pneumoniae* strains irrespective of their capsular type. Immunization with the pneumococcal cytoplasmic protein pneumolysin can protect mice against pneumococcal infection (87). This protection is probably due to neutralization of free toxin released from the pneumococcus during autolysis. Anti-pneumolysin antibodies are not expected to promote phagocytosis. A nontoxic variant of pneumolysin may be a good component for future pneumococcal protein and/or conjugate vaccines. PspA is a protection-eliciting surface protein found on all pneumococci. Although the primary structure of PspA is highly variable (30, 113), the protein is able to elicit cross-protection (73). Therefore, PspA is presumed to be a good vaccine candidate. Immunization with autolysin (11), neuraminidase (68) and PsaA (108) also clearly confer protection in animal models. However, since PsaA might be involved in triggering autolysin, loss of function of this protein may promote penicillin tolerance (83). To date, no protection could be demonstrated using purified hyaluronidase as an immunogen. Furthermore, the pneumococcal IgA1 protease has shown to be antigenically diverse making it a less attractive vaccine candidate (69). Due to the genetic plasticity of the pneumococcus, the optimal protein-based vaccine is proposed to contain multiple protein antigens.

Scope of this thesis

This thesis focuses on molecular epidemiological aspects of infection by *S. pneumoniae* and the identification of pneumococcal proteins involved in the pathogenesis of infections.

The epidemiological dynamics of penicillin-nonsusceptible *S. pneumoniae* isolates from 16 different countries were investigated (Chapter 2). In addition, nation-wide molecular epidemiological surveys of penicillin-nonsusceptible pneumococci from Poland and Thailand were performed (Chapters 3 & 4). The molecular mechanisms of penicillin resistance in The Netherlands were studied in detail by sequence analysis of *pbp* fragments (Chapter 5). A molecular epidemiological survey was also performed on penicillin-susceptible invasive *S. pneumoniae* isolates from The Netherlands. In the latter study, the genetic relatedness within serotypes was investigated, and in addition, the international molecular epidemiological characteristics of serotype 3 isolates were identified (Chapter 6). The effect of immune-protective antibodies raised against large-scale vaccination on natural competence of *S. pneumoniae* was investigated, and the consequences for the long-term efficacy of current vaccination strategies are discussed (Chapter 7). To gain insight into the pathogenesis of pneumococcal disease, the molecular changes that occur during phase variation were investigated. To this purpose, protein expression patterns of pneumococcal phenotypic variants were compared, and differentially expressed proteins were identified and characterized (Chapter 8). Interestingly, one of these proteins, pyruvate oxidase, was also demonstrated to play a role in the inhibition of other respiratory tract pathogens (Chapter 9). A second protein, the putative proteinase maturation protein A, PpmA, was also independently identified in another study as a conserved surface-exposed virulence factor with potential to elicit protective immune responses (Chapter 10).

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Chapter 2

Penicillin-resistant *Streptococcus pneumoniae*: an international molecular epidemiological study

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SUMMARY

An international study was undertaken to investigate the epidemiological dynamics of penicillin-resistant pneumococci. We compared the molecular epidemiological characteristics of 316 penicillin-resistant isolates, originating from The Netherlands, Thailand, the United States, Spain, Greece, Poland, Cuba, Germany, Finland, United Kingdom, Iceland, South Africa, Hungary, Portugal, Croatia and the Czech Republic. A total of 132 distinct restriction fragment end labeling (RFEL) types were observed. Thirty-six genetic types were shared by two or more strains (clusters). Seven RFEL clusters consisted of strains originating from different countries, demonstrating international dissemination of penicillin-resistant pneumococci. The two most predominant RFEL types corresponded with the pandemic clones 23F and 9V, and were found in 10 and six different countries, respectively. This clearly demonstrates the pandemic behavior of these two clones. Fifteen out of the 36 RFEL clusters contained two or more serotypes. This finding indicates frequent horizontal transfer of capsular genes. Within distinct RFEL types identical penicillin-binding protein (PBP) genotypes were often observed, demonstrating a high frequency of horizontal DNA transfer of penicillin resistance genes. The most predominant PBP type comprised 40% of the entire collection. This PBP type was found in 20 distinct RFEL types and was observed in 11 countries. The vast majority of the strains belonging to the pandemic clones 23F and 9V shared this predominant PBP type. We hypothesize that the clones 23F and 9V are responsible for the worldwide increase of penicillin resistance, since they serve as an important genetic reservoir for susceptible pneumococci to acquire penicillin resistance.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) continues to be a common cause of serious and life-threatening infections such as pneumonia, bacteremia and meningitis, and of noninvasive infections such as otitis media and sinusitis. In the late 1970s and 1980s, the incidence of penicillin-resistant pneumococci has increased in Western countries, particularly in Spain, reaching levels up to 50% (6, 12, 14, 22). An epidemiological study in the United States in 1995 has demonstrated that 25% of invasive pneumococci were intermediate-level or high-level resistant to penicillin (11). The emergence of high-level resistance to penicillin, particularly in combination with other resistance determinants, possesses serious problems for

the institution of adequate antimicrobial therapy.

The high incidence of infections caused by pneumococci and the emergence of drug-resistant isolates form major objectives for molecular epidemiological surveillance. In order to obtain meaningful epidemiological information, genetic markers are needed that reflect an adequate rate of genetic rearrangements over time. Various pheno- and genotypical methods have been developed to assist in epidemiological investigations. These methods include serotyping, multilocus enzyme electrophoresis, pneumococcal surface protein A typing, penicillin-binding protein (PBP) typing, and DNA fingerprint methods such as pulsed-field gel electrophoresis, ribotyping and DNA fingerprinting of the PBP genes. We have recently investigated the usefulness of various DNA fingerprint methods to study the molecular epidemiology of pneumococcal infections. The potentials of (i) ribotyping, (ii) BOX fingerprinting using the BOX repetitive sequence of *S. pneumoniae* as a DNA probe, (iii) polymerase chain reaction fingerprinting using a primer homologous to the enterobacterial repetitive intergenic consensus sequence or homologous to the pneumococcal BOX repetitive sequence, (iv) pulsed-field gel electrophoresis of large-size DNA fragments, and (v) restriction fragment end labeling (RFEL) to detect restriction fragment length polymorphism of small-size DNA fragments have been compared (10, 30). Although the discriminatory power of the DNA fingerprint techniques differs significantly, the deduced genetic clustering of the pneumococcal strains is comparable. The ease to perform computerized analysis and the potential to create reliable fingerprint libraries also differs significantly. Ribotyping, BOX fingerprinting and RFEL analysis are very suitable for computerized analysis of the fingerprints. RFEL analysis, a technique that has also proved to be relatively stable over time (24), is currently routinely used in our laboratory to generate a data library of pneumococcal DNA fingerprints (10).

Several examples of international spread of resistant pneumococcal clones have been reported: (i) Soares et al. have documented the spread of a multiresistant clone of serotype 6B from Spain to Iceland in the late 1980s (28). This has resulted in an epidemic of this clone, which was isolated with a frequency up to 12% already in 1992 (13). (ii) In 1991, Munoz and colleagues have reported evidence for the intercontinental spread of a multiresistant clone of *S. pneumoniae* serotype 23F from Spain to the United States (16). This clone has subsequently disseminated through the United States (15). (iii) Gasc et al. have reported in 1995 the spread of a penicillin-resistant pneumococcal clone of serogroup 9 from Spain to France (5). The latter clone has also recently been observed in Germany (19). Besides the

international spread of the clones 6B, 23F and 9V, novel penicillin-resistant and multiresistant clones have been reported in former Czechoslovakia, Spain, Japan and South Africa that tend to spread in an epidemic manner within these countries (2, 21, 26, 32).

In the present study, we investigated the international epidemiological dynamics of penicillin-resistant pneumococci. For this purpose, we compared the molecular epidemiological characteristics of 316 penicillin-resistant isolates, originating from 16 distinct countries including The Netherlands, Thailand, the United States, Spain, Greece, Poland, Cuba, Germany, Finland, United Kingdom, Iceland, South Africa, Hungary, Portugal, Croatia and the Czech Republic.

MATERIALS AND METHODS

Bacterial isolates. Penicillin-resistant pneumococcal isolates (n = 316) were collected in 16 different countries. A total of 188 penicillin-resistant isolates [minimum inhibitory concentration (MIC) ≥0.1 mg/L] were collected in The Netherlands. Thirty-nine Dutch medical microbiology laboratories participated in this study, and sent all penicillin-resistant pneumococci isolated between March 1995 and March 1997 to our laboratory. These 39 laboratories offer microbiological services to the majority of the Dutch hospitals. The participating hospitals provide medium-, high- and intensive-care facilities, and are distributed all over The Netherlands. The laboratories performed susceptibility testing on all pneumococcal isolates, and all penicillin-resistant pneumococci were included in the study. Duplicate isolates from patients were excluded. The clinical origin of the Dutch isolates was: sputum (n = 126), nasopharynx (n = 21), nose (n = 19), blood (n = 7), cerebrospinal fluid (n = 3), pus (n = 3), bronchial secretion fluid (n = 3), ear (n = 2), vagina (n = 1), hypopharynx (n = 1) and conjunctiva (n = 1). One pneumococcal isolate was of unknown clinical origin.

Fifty-three penicillin-resistant isolates from Thailand were collected between February 1993 and May 1994 in six hospitals located in five distinct regions of the country. The isolates all originated from the nasopharynx of single individuals under the age of five with acute respiratory infections.

Nine penicillin-resistant isolates were collected between February 1994 and September 1995 in Kyriakou Children's Hospital in Athens, Greece. These strains originated from patients under the age of 14 years (mean age 2.75 years), suffering from otitis media (n = 5), rhinitis (n = 2), bronchiolitis (n = 1) or bacteremia (n = 1).

Five penicillin-resistant isolates were collected in Cuba. The isolates all originated from the nasopharynx of single individuals under the age of five with respiratory infections.

We further received five penicillin-resistant pneumococci from Poland, which were isolated in three distinct hospitals from children suffering from otitis media (n = 1), sinusitis (n = 1) or pharyngitis (n = 3).

In order to expand our pneumococcal collection with DNA fingerprints from penicillin-resistant strains, we asked various colleagues to provide us with clinical isolates. These strains originated from the United States [n = 10, R. R. Facklam (8); n = 9, H. Faden (25); n = 4, A. Tomasz; n = 1, F. Tenover], Spain [n = 3, R. Hakenbeck (19); n = 10, J. Casal], Germany [n = 4, R. Hakenbeck (19)], Finland [n = 3, R. Hakenbeck (23)], United Kingdom [n = 3, L. M. C. Hall (7)], Iceland (n = 3, K. Kristinsson), South Africa [n = 2, R. Hakenbeck (23)], Hungary [n = 1, R. Hakenbeck (19)], Portugal (n = 1, A. Tomasz), Croatia (n = 1, A. Tomasz), and the Czech Republic [n = 1, R. Hakenbeck (19)].

Biochemical characterization, serotyping and susceptibility testing. Species identification of the *S. pneumoniae* isolates was performed using optochine susceptibility and bile solubility testing (17). Pneumococci were serotyped on the basis of capsular swelling (Quellung reaction) observed microscopically after suspension in antisera prepared at Statens Serum Institut, Copenhagen, Denmark (4).

The MICs of the pneumococcal strains were determined by agar dilution. The MIC was defined as the lowest concentration of the antimicrobial agent preventing visible growth. For this purpose, serial ^{2}log concentrations of antibiotics were prepared in IsoSensitest agar (Oxoid, Unipath Ltd., Basingstoke, United Kingdom), supplemented with 5% horse blood. The pneumococcal isolates were removed from storage at -70°C, and sub-cultured at 37°C on Columbia agar (Oxoid) supplemented with 5% sheep blood using 5% CO_2. Bacterial suspensions were prepared in 0.9% NaCl from 24-h agar cultures, and adjusted to a McFarland turbidity of 0.5. Suspensions were further diluted (1:10) in saline. The inocula were applied on the test plates using a multipoint inoculator, resulting in about 10^4 colony forming units per spot. MIC values were read after 24 h of incubation at 37°C using 5% CO_2.

The antimicrobial agents tested were: penicillin G (Sigma Chemical Co., St. Louis, MO), erythromycin (Abbott Lab. Ltd, Queenborough, Kent, United Kingdom), doxycycline (Pfizer S.A., Brussels, Belgium), vancomycin (Eli Lilly Indianapolis, In.), rifampicin (Sigma), cotrimoxazole, i.e., the combination (1:19) of trimethoprim (Sigma) and sulphamethoxazole

(Sigma), and ciprofloxacin (Bayer, Wuppertal, Germany). Breakpoints of the antibiotics to discriminate between susceptible and nonsusceptible strains were used according to the NCCLS guidelines for susceptibility testing (18).

RFEL analysis. Typing of pneumococcal strains by RFEL was performed as described by Van Steenbergen et al. (31), and adapted by Hermans et al. (10). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end-labeled at 72°C with [α-^{32}P]dATP using Taq DNA polymerase (Goldstar, Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured, and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. Subsequently, the gel was transferred onto filter paper, vacuum-dried (HBI, Saddlebrook, NY), and exposed for variable periods at room temperature to ECL Hyperfilms (Amersham, Bucks, United Kingdom).

PBP-genotyping. Genetic polymorphism of the PBP genes *pbp1a*, *pbp2b* and *pbp2x* was investigated by RFLP analysis. For this purpose, we amplified the genes by PCR, and analyzed the digested DNA products by agarose gel electrophoresis. PCR-amplification of the PBP genes was performed in a 50-µl PCR buffer system containing 75 mM Tris-HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) of Tween 20, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of the individual primers, 0.5 units of DNA polymerase (Eurogentec) and 10 ng/µl of purified chromosomal DNA. Cycling was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA), and consisted of the following steps: pre-denaturation at 94°C for 1 min, 30 cycles of 1 min 94°C, 1 min 52°C, 2 min 72°C, and final extension at 72°C for 3 min. The primers used to amplify the PBP 1a, 2b and 2x genes were described previously (1, 3, 16). The amplification products (5 µl) were digested by restriction endonuclease *Hinf*I, and separated by electrophoresis in 2.5% agarose gels containing 0.5 x TBE and 0.1 µg/ml of ethidium bromide (5 mm thick; Agarose MP, Boehringer Mannheim, Almere, The Netherlands). Gels were run in 0.5 x TBE containing 0.1 µg/ml of ethidium bromide at a constant current of 20 mA for 4 h. Prior to electrophoresis, samples were mixed with a 5 x concentrated layer mix consisting of 50% glycerol in water and 0.8 mg bromo phenol blue per ml. Gels were photographed with a Polaroid MP4 Landcamera and Polaroid 667 films. The different PBP genotypes are represented by a three-number code (i.e., 06-05-19), referring at the RFLP patterns of the PBP genes 1a (6), 2b (5) and 2x (19), respectively.

Computer-assisted analysis of the DNA fingerprints. The RFEL types were analyzed using the Windows version of the Gelcompar software version 4 (Applied Maths, Kortrijk, Belgium) after scanning the RFEL autoradiograms using the Image Master DTS (Pharmacia Biotech, Uppsala, Sweden). For this purpose, the DNA fragments in the molecular weight range of 160 to 400 base pairs were explored. The fingerprints were normalized using pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the fingerprints was performed by unweighted pair group method using arithmetic averages (UPGMA) (20), and using the Jaccard similarity coefficient applied to peaks (27). Computer-assisted analysis, and methods and algorithms used in this study were carried out according to the instructions of the manufacturer of Gelcompar. A tolerance of 1.5% in band positions was applied during comparison of the fingerprint patterns. Identical DNA types were arbitrarily defined as RFEL homologies higher than 95%. A genetic cluster was defined being a genotype (RFEL or PBP type) that was shared by two or more pneumococcal strains. The degree of genetic clustering was defined as the percentage of strains displaying genotypes (RFEL or PBP types) that were observed twice or more.

RESULTS AND DISCUSSION

All 316 penicillin-resistant pneumococcal strains were analyzed by serotyping, RFEL and PBP genotyping, and the resistance patterns were determined. The results are summarized in Fig. 1. One hundred and thirty-two distinct RFEL types were observed, representing 36 genetic clusters (RFEL types shared by two or more strains) and 96 unique RFEL types. The largest collections of strains originated from The Netherlands (n = 188) and Thailand (n = 53), and demonstrated 76% and 68% of genetic clustering, respectively. The degree of clustering was much higher among the penicillin-resistant strains from both countries compared with a group of 153 penicillin-susceptible strains isolated in The Netherlands in 1994, which displayed only 33% of genetic clustering (9). These data clearly demonstrate that the transmission behavior of these strains is comparable in both countries. We have recently investigated in a one-year survey the epidemiological characteristics of penicillin-resistant pneumococci in The Netherlands. The data obtained from this study have demonstrated that multiple clones of penicillin-resistant pneumococci are frequently introduced in this low-prevalence country. Some of these isolates are able to spread among the population in and

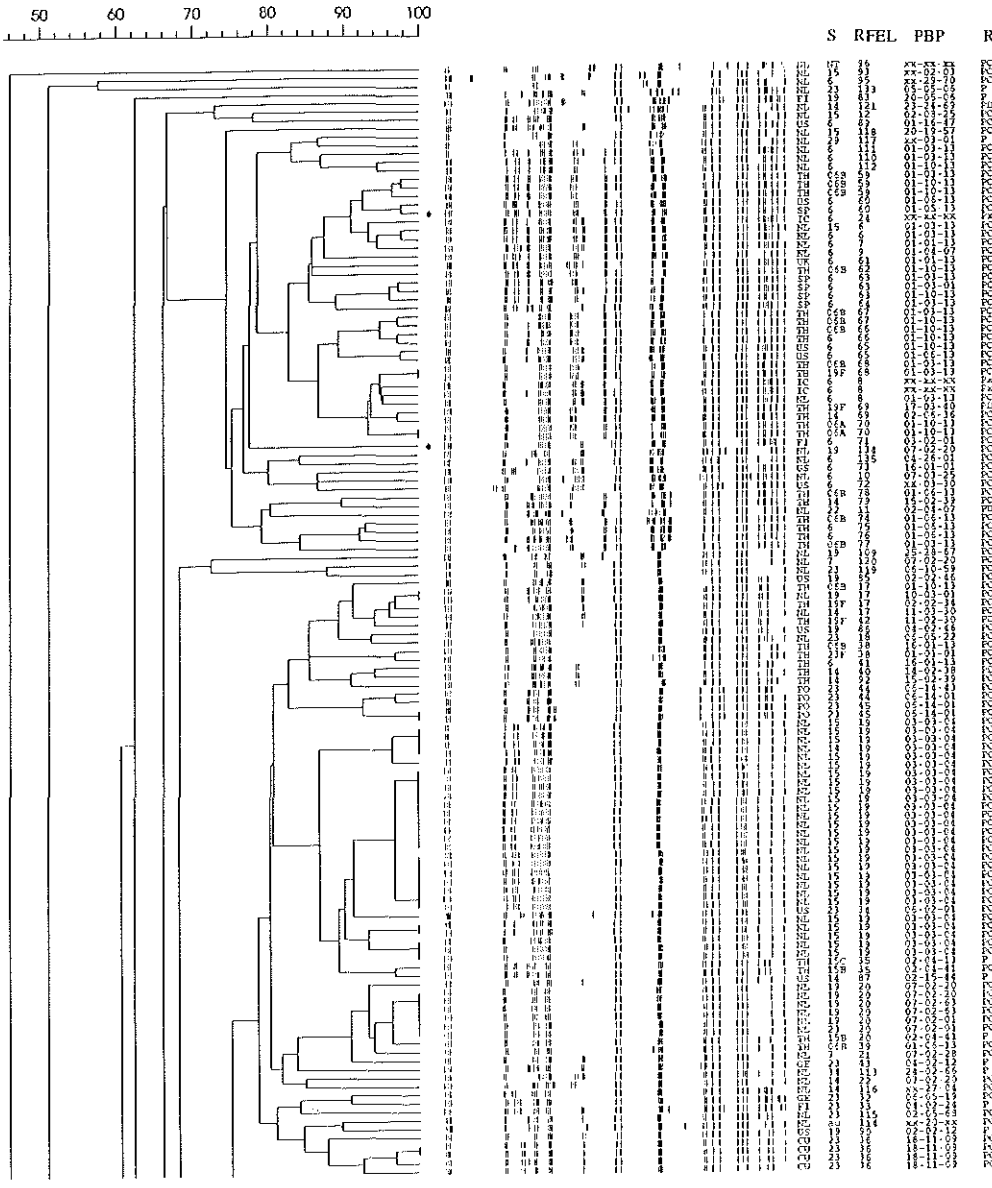
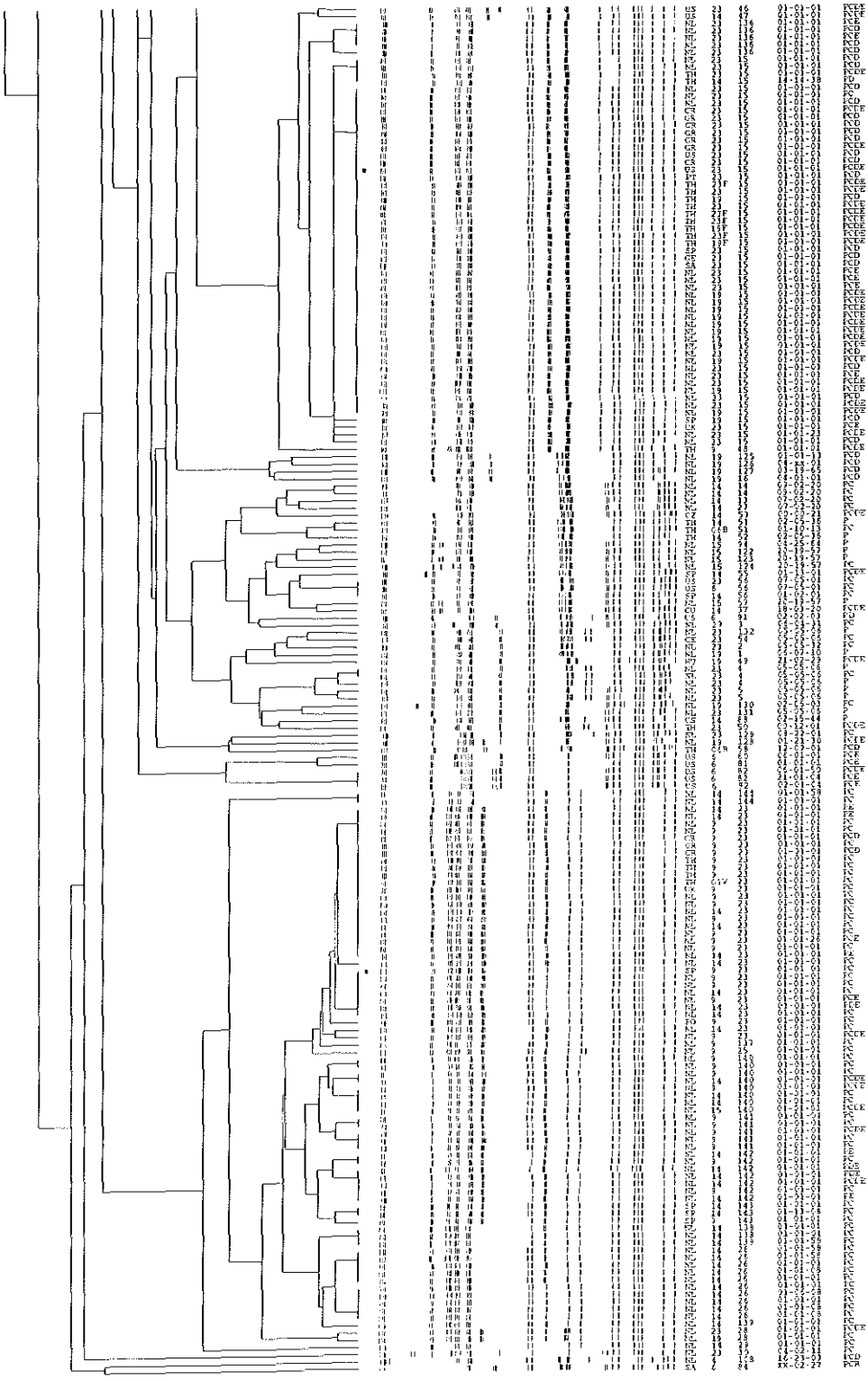


Fig. 1. Dendrogram of the RFEL banding patterns of 316 penicillin-resistant pneumococci originating from The Netherlands (NL, *n* = 188), Thailand (TH, *n* = 53), the United States (*n* = 24), Spain (SP, *n* = 13), Greece (GR, *n* = 9), Poland (PO, *n* = 5), Cuba (CU, *n* = 5), Germany (GE, *n* = 4), Finland (FI, *n* = 3), United Kingdom (UK, *n* = 3), Iceland (IC, *n* = 3), South Africa (SA, *n* = 2), Hungary (HU, *n* = 1), Portugal (PT, *n* = 1), Croatia (CR, *n* = 1) and the Czech Republic (CZ, *n* = 1). Characters in brackets indicate serogroup (S), RFEL type (RFEL), PBP type (PBP) and resistance pattern (R). The arbitrary cut-off value for an identical RFEL type is 95%, except for RFEL type 19 (for details, see 9). The previously described pandemic clones 23F (■; 29), 9V (●; 19) and 6B (♦; 19) are also depicted. P, penicillin G; C, cotrimoxazole; D, doxycycline; E, erythromycin; R, rifampicin.; xx, unknown PBP type; NT, non-typable.



outside hospitals, in particular in sub-populations with a high risk for pneumococcal infections and a high consumption of antibiotics (9).

Among the 316 penicillin-resistant pneumococci, 236 strains (75%) were intermediately resistant to penicillin and 77 (24%) displayed high-level resistance. Forty-seven percent of the penicillin-resistant strains were co-resistant to erythromycin, 56% to doxycycline and 87% to cotrimoxazole. All isolates were in vitro susceptible to vancomycin and ciprofloxacin. The resistance patterns of the penicillin-resistant pneumococci originating from The Netherlands are summarized in Table 1. Comparison of the pneumococci with penicillin MIC values <1 μg/ml and those with MIC values ≥1 μg/ml clearly demonstrated that the penicillin-resistant pneumococci with MIC values ≥1 μg/ml were more frequently multiply resistant to four antibiotics (resistance pattern PCDE). In addition, the degree of genetic clustering using both RFEL typing and PBP typing is also higher among the penicillin-resistant isolates displaying MICs ≥1 μg/ml. These data suggest that the epidemic behavior of the penicillin-resistant strains is enhanced by increasing levels of penicillin resistance. Moreover, we hypothesize that horizontal co-transfer of antibiotic resistance genes other than PBP genes occurs frequently among pneumococci with high-level resistance to penicillin.

Table 1. Resistance patterns of penicillin-resistant pneumococci in The Netherlands (n = 188).

	MIC for penicillin (μg/ml)					
	0.1 - 1			> 1		
		genetic clustering (%)			genetic clustering (%)	
Resistance pattern	Number of strains	RFEL	PBP	Number of strains	RFEL	PBP
P	16	25	56	1		
PC	18	0	39	45	87	91
PD	2	0	0	0		
PE	0			6	100	100
PCD	10	0	30	11	91	91
PCE	0			6	83	83
PDE	1			1		
PCDE	10	30	20	61	87	87

P, penicillin G; C, cotrimoxazole; D, doxycycline; E, erythromycin.

Seven out of the 36 RFEL clusters consisted of strains originating from different countries (Table 2). The strains displaying the two predominant RFEL types 15 and 23 were found in 10 and six different countries, respectively. These data demonstrate the pandemic spread of these two genotypes.

Table 2. Genotypic and phenotypic properties of penicillin-resistant pneumococci sharing identical RFEL types.

RFEL type[a]	Number of strains	Number of countries	Serogroup (number of strains)	RFEL type[a]	Number of strains	Number of countries	Serogroup (number of strains)
15[b]	53	10	23 (37)	138	3	1	14 (3)
			19 (15)				
			14 (1)				
23[c]	32	6	09 (22)	6	2	1	06 (1)
			14 (10)				15 (1)
19	27	1	15 (26)	28	2	1	19 (1)
			14 (1)				23 (1)
26	10	1	14 (10)	38	2	1	06 (1)
							23 (1)
140	8	1	09 (4)	51	2	1	06 (1)
			14 (4)				14 (1)
20	7	2	19 (5)	68	2	1	06 (1)
			15 (1)				19 (1)
			23 (1)				
142	7	1	14 (5)	69	2	1	14 (1)
			09 (2)				19 (1)
136	5	1	23 (5)	60	2	2	06 (2)
141	5	1	09 (5)	5	2	1	23 (2)
17	4	2	19 (2)	14	2	1	14 (2)
			14 (1)				
			06 (1)				
36	4	1	23 (4)	35	2	1	15 (2)
56	3	2	06 (1)	44	2	1	23 (2)
			14 (1)				
			23 (1)				
143	3	1	14 (2)	45	2	1	23 (2)
			09 (1)				
8	3	2	06 (3)	65	2	1	06 (2)
4	3	1	23 (3)	66	2	1	06 (2)
59	3	1	06 (3)	67	2	1	06 (2)
63	3	1	06 (3)	70	2	1	06 (2)
82	3	1	06 (3)	144	2	1	14 (2)

[a] RFEL types displayed by 2 or more strains (RFEL clusters) were exclusively included.
[b] RFEL type 15 represents pandemic clone 23F (16).
[c] RFEL type 23 represents pandemic clone 9V (5).

Fifteen out of the 36 RFEL clusters harbored two or more serogroups (Table 2). This indicates frequent horizontal transfer of capsular genes among pneumococci. The high frequency of capsular transfer may have consequences with regard to the outcome of current vaccine strategies, which focus entirely on the use of capsular polysaccharides representing a restricted number of capsular types. The use of multivalent conjugate vaccines may shift the capsular distribution towards capsular types that are not present in these vaccines. Such a shift might be enhanced by the frequent horizontal exchange of capsular genes.

All 316 penicillin-resistant pneumococci were analyzed by PBP genotyping. Ninety-two distinct PBP types were observed, representing 22 PBP clusters (PBP genotypes shared by

two or more strains) and 70 unique PBP types (Fig. 1, Table 3). No overlap was seen between the PBP types of the penicillin-resistant pneumococci and more than 200 susceptible isolates (data not shown). Within distinct RFEL types, identical PBP types were often observed. Forty percent of the collection of 316 penicillin-resistant pneumococci displayed PBP type 01-01-01. This predominant PBP type was observed in 11 countries and displayed by 20 distinct RFEL types (Table 3). This observation suggests frequent horizontal exchange of PBP genes, which has resulted in the spread of PBP type 01-01-01 among various pneumococcal RFEL types.

Within the group of 316 penicillin-resistant strains, the previously described pandemic clones 23F (16), 9V (5) and 6B (28) were also included (Fig. 1). The predominant RFEL type 15, observed in 10 countries, was identical with the RFEL type of the pandemic clone 23F, whereas RFEL type 23, observed in six countries, was displayed by the pandemic clone 9V, respectively. Interestingly, all isolates from Greece ($n = 9$) matched either with clone 23F ($n = 6$) or with clone 9V ($n = 3$), indicating the significance of both clones in this country (Fig. 1). In addition, the vast majority of the strains belonging to the pandemic clones 23F and 9V shared the predominant PBP type 01-01-01 (Table 3). We hypothesize that the predominant pandemic clones 23F and 9V are primarily responsible for the increase of penicillin-resistance worldwide, because they are an important penicillin resistance reservoir for susceptible pneumococci.

The previously identified pandemic 6B-type strains which displayed genetically related multilocus enzyme electrophoresis patterns (19), only shared 79% of the RFEL DNA bands. Interestingly, the two RFEL types belonged to a genetic family that branched at 75% genetic relatedness (Fig. 1). Within this genetic family, serogroup 6 was displayed by 82% of the members. Only 27% of the serogroup 6 strains did not match within this family. These data indicate that most of the serogroup 6 strains are clonally related, and linked within a single genetic family. Genetic polymorphism observed by PBP typing was also restricted within this family, and *pbp2x* type 13 was predominantly (68%) and almost exclusively present among the family members (Fig. 1).

This study aimed to identify the clones of penicillin-resistant pneumococci that are currently displaying pandemic behavior. Our data clearly show the importance of the pandemic clones 23F and 9V, as they were detected in 10 and six out of the 16 countries, respectively. In addition, serogroup 6 strains were also frequently observed. However, the genetic homogeneity of these strains is reduced. This finding is consistent with the observations of

Table 3. Genotypic and phenotypic properties of penicillin-resistant pneumococci sharing identical PBP types.

PBP type[a] (*1a-2b-2x*)	Number of strains	Number of countries	RFEL type (number of strains)	Serogroup (number of strains)
01-01-01[b]	127	11	15 (51)	23 (44)
			23 (31)	09 (36)
			140 (8)	19 (16)
			142 (7)	14 (29)
			136 (5)	06 (1)
			141 (5)	15 (1)
			26 (4)	
			28 (2)	
			138 (2)	
			143 (2)	
			25 (1)	
			29 (1)	
			38 (1)	
			46 (1)	
			47 (1)	
			48 (1)	
			81 (1)	
			137 (1)	
			139 (1)	
			144 (1)	
03-03-04	27	1	19 (27)	15 (26)
				14 (1)
01-10-13	13	4	59 (2)	06 (13)
			66 (2)	
			70 (2)	
			17 (1)	
			51 (1)	
			62 (1)	
			63 (1)	
			65 (1)	
			67 (1)	
			112 (1)	
01-03-13	12	3	06 (2)	06 (10)
			68 (2)	15 (1)
			08 (1)	19 (1)
			59 (1)	
			63 (1)	
			64 (1)	
			67 (1)	
			77 (1)	
			110 (1)	
			111 (1)	

Table 3. Genotypic and phenotypic properties of penicillin-resistant pneumococci sharing identical PBP types (cont'd).

PBP type[a] (*1a-2b-2x*)	Number of strains	Number of countries	RFEL type (number of strains)	Serogroup (number of strains)
05-05-06	9	2	04 (3) 05 (2) 54 (1) 131 (1) 132 (1) 133 (1)	23 (9)
01-06-13	7	2	39 (1) 60 (1) 65 (1) 74 (1) 75 (1) 76 (1) 78 (1)	06 (7)
07-02-20	7	1	20 (2) 13 (1) 22 (1) 27 (1) 120 (1) 134 (1)	14 (3) 19 (3) 07 (1)
20-19-57	5	1	57 (1) 118 (1) 122 (1) 123 (1) 124 (1)	15 (5)
01-01-58	4	1	26 (2) 138 (1) 144 (1)	14 (4)
18-11-09	4	1	36 (3) 37 (1)	23 (4)
01-01-13	3	2	07 (1) 61 (1) 125 (1)	06 (2) 09 (1)
06-14-01	3	1	44 (1) 45 (2)	23 (3)
01-01-08	3	1	26 (3)	14 (3)
02-04-41	2	1	20 (1) 35 (1)	15 (2)
02-15-14	2	1	87 (1) 88 (1)	14 (2)
07-02-01	2	1	20 (2)	19 (1) 23 (1)

Table 3. Genotypic and phenotypic properties of penicillin-resistant pneumococci sharing identical PBP types (cont'd).

PBP type[a] (*1a-2b-2x*)	Number of strains	Number of countries	RFEL type (number of strains)	Serogroup (number of strains)
07-05-01	2	1	56 (2)	06 (1) 23 (1)
15-02-39	2	1	40 (1) 79 (1)	14 (2)
16-01-13	2	1	51 (1) 52 (1)	06 (2)
02-05-36	2	2	51 (2)	14 (2)
07-02-63	2	1	20 (2)	19 (2)
09-02-20	2	1	14 (2)	14(2)

[a] RFEL types displayed by 2 or more strains (RFEL clusters) were exclusively included.
[b] Includes the vast majority of the strains belonging to the pandemic clones 23F (16) and 9V (5).

Harakeh et al. (8) who have demonstrated genetic heterogeneity within serotype 6B pneumococci isolated in the United States. Using a genetic relatedness ≥75%, serogroup 6 strains were observed in seven countries. These observations suggest that the introduction and subsequent pandemic spread of clone 6B has occurred earlier in the antibiotic era compared with the international clones 23F and 9V. The contribution of clone 6B to the global increasing prevalence of penicillin-resistant pneumococci is at present overruled by the rapidly spreading clones 23F and 9V.

Detection and reduction of transmission of alarming drug-resistant pneumococci that are currently spreading all over the world is an important goal in the battle against pneumococcal disease. Obviously, restrictive use of antibiotics remains the major defense against the epidemic dissemination of such strains. In addition, the emergence of multidrug-resistant pneumococci increases the need for vaccination of patients at risk for pneumococcal disease such as the elderly, and the importance to improve conjugate vaccines to efficiently protect young children. Detailed studies on the epidemiology and epidemic behavior of (multi)resistant pneumococci will assist to identify emerging clones. To this respect, close collaboration between the laboratories sharing interests in pneumococcal molecular epidemiology is of utmost importance. Extensive collaboration can be facilitated by the establishment of a freely accessible electronic network. Such network can be used to exchange information on technical aspects of DNA fingerprinting, aimed to standardize the

methodological procedures. In addition, the network can be used to construct and distribute an international data library containing DNA fingerprints of (multi)resistant pneumococcal strains. Such approach will facilitate adequate worldwide monitoring of the epidemiology of emerging (multi)resistant pneumococcal strains.

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Chapter 3

Multidrug-resistant *Streptococcus pneumoniae* in Poland: identification of emerging clones

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SUMMARY

Penicillin resistance among *Streptococcus pneumoniae* isolates has rapidly emerged in Poland during the last decade and has reached prevalence levels of up to 14.4% in 1997. In order to investigate the nature of this increase, a molecular epidemiological analysis of penicillin-nonsusceptible multidrug-resistant pneumococci isolated in 1995 and 1996 was conducted. Thirty-seven patients who suffered mainly from upper respiratory tract infections and pneumococcal pneumonia were enrolled in this study. The medical centers to which the patients were admitted were located in 16 Polish towns across the country. Eight distinct BOX PCR types were observed, representing 14 subtypes. Restriction fragment end labeling (RFEL) analysis divided the pneumococcal strains into 16 distinct types. By combining the BOX PCR and RFEL data, four genetically distinct clusters of strains were identified. Two clusters represented the genetic clones 23F and 9V, which have recently emerged all over the world. The two other genetic clusters, which represented serotypes 23F and 6B, clearly predominated in the analyzed collection of Polish penicillin-nonsusceptible pneumococcal strains. Since the latter clusters did not match any of the 133 RFEL types of penicillin-nonsusceptible pneumococci collected in 15 other countries, their Polish clonal origin is most likely.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) continues to be a common cause of serious and life-threatening infections, such as pneumonia, bacteremia, and meningitis, and of non-invasive infections, such as otitis media and sinusitis (3). In addition, pneumococci are often part of the normal nasopharyngeal flora. Especially in young children, pneumococcal colonization often occurs. Colonization is an important risk factor: children colonized with *S. pneumoniae* more often develop acute otitis media than children who are not colonized (9, 18, 33, 43).

Until the 1960s, pneumococci were considered uniformly susceptible to penicillin, and sensitivity tests were hardly ever performed. In 1967, the first isolation of penicillin-resistant pneumococci was reported in Australia (12). Subsequently, penicillin-resistant pneumococci have been isolated in Papua New Guinea and South Africa (20, 22, 31). In the late 1970s and 1980s, rates of resistance, including multiple resistance, have increased in Western countries, particularly in Spain, with resistance levels of up to 50% (11, 20, 22, 31). A recent

epidemiological study performed in the United States has demonstrated that 25% of invasive pneumococci are penicillin-nonsusceptible (17). The emergence of high-level resistance to penicillin, particularly in combination with other resistance determinants, is a serious threat for current treatment strategies.

Several investigators have reported the international spread of multiply-resistant pneumococcal clones. Soares and coworkers have reported the spread of a drug-resistant clone of serotype 6B from Spain to Iceland in the late 1980s (37). This has resulted in an epidemic of this clone, which was isolated with frequencies of up to 12% in 1992 (21). In 1991, Munoz and colleagues reported evidence for the intercontinental spread of a multiply resistant clone of *S. pneumoniae* serotype 23F from Spain to the United States (24). This clone has subsequently disseminated through the United States (23). Finally, in 1995 Gasc and colleagues reported the spread of a multiply-resistant pneumococcal clone of serogroup 9 from Spain to France (10). The international clones 23F and 9V have been identified in many countries in different parts of the world (14, 28). Besides the international spread of the clones 6B, 23F, and 9V, novel penicillin-resistant and multiply-resistant clones that tend to spread in an epidemic manner have been observed in the former Czechoslovakia, Spain, Japan, and South Africa (4, 30,34, 42).

Penicillin resistance among pneumococcal isolates has rapidly emerged in Poland during the last decade. The percentage of penicillin-nonsusceptible strains has increased from 0 to 3% in 1990 to 1993 (1, 19) to 14.3% in 1996 (39) and 14.4% in 1997 (38). In order to identify the nature of the increase in the prevalence of penicillin-nonsusceptible pneumococci in Poland, a molecular epidemiological study was conducted. To this end, strains were isolated in 1995 and 1996 from 37 patients who suffered mainly from upper respiratory tract infections and pneumococcal pneumonia and who were diagnosed in 19 different clinical centers located in 16 towns across the country. The pneumococcal isolates were characterized by BOX PCR typing, restriction fragment end labeling (RFEL) analysis, penicillin-binding protein (PBP) genotyping, serotyping, and drug susceptibility testing.

MATERIALS AND METHODS

Bacterial isolates. Penicillin-nonsusceptible *S. pneumoniae* strains were isolated from 37 Polish patients in 1995 and 1996. A single pneumococcal isolate per patient per infection was analyzed. Bacteriological diagnosis was carried out in 19 distinct medical centers. These

centers were located in 16 Polish towns across the country (Fig. 1). The clinical, bacteriological, and demographic parameters are listed in Table 1.

Fig. 1. Map of Poland. The medical centers collaborating in this study are as follows: 1, Mother and Child Institute, Warsaw; 2, Health Care Center Praga-Polnoc, Warsaw; 3, Nieklanska Street Hospital, Warsaw; 4, Sienna Street Hospital, Warsaw; 5, Health Care Center, Kolobrzeg; 6, Regional Hospital, Gdansk; 7, Health Care Center, Tczew; 8, Health Care Center, Braniewo; 9, Regional Children Hospital, Olsztyn; 10, Regional Hospital, Suwalki; 11, Medical University, Bydgoszcz; 12, Health Care Center, Gorzow Wielkopolski; 13, Health Care Center, Czestochowa; 14, Health Care Center, Kedzierzyn Kozle; 15, Jan Bober Center for Microbiology and Autovaccines, Krakow; 16, Health Care Center, Myslenice; 17, Health Care Center, Mielec; 18, Health Care Center, Sanok; and (19), Regional Hospital, Lomza.

Biochemical characterization, serotyping, and susceptibility testing. The isolates were determined to be *S. pneumoniae* by investigating their optochin susceptibility and bile solubility (25). All pneumococci were serotyped in the Sera and Vaccines Central Research Laboratory in Warsaw on the basis of capsular swelling (Quellung reaction) observed microscopically after suspension in antisera prepared at Statens Serum Institut, Copenhagen, Denmark (8).

The susceptibilities of pneumococcal strains were determined in the Sera and Vaccines Central Research Laboratory in Warsaw according to the guidelines of the National Committee for Clinical Laboratory Standards (26) by the broth microdilution method in cation-adjusted Mueller-Hinton II broth (Beckton Dickinson, Oxford, United Kingdom) supplemented with 5% lysed horse blood. The following panel of antimicrobial agents was tested: penicillin (Polfa, Poznan, Poland), amoxicillin (SmithKline Beecham, Brentford, United Kingdom), cefotaxime (Roussel, Paris, France), doxycycline (Polfa), chloramphenicol (Polfa), erythromycin (Polfa), lincomycin (Upjohn, Kalamazoo, Mich.), vancomycin (Eli Lilly, Indianapolis, Ind.), rifampin (Polfa), ofloxacin (Hoechst-Roussel, Kansas City, Kans.), sparfloxacin (Rhone-Poulenc, Courbevoie cedex, France), and cotrimoxazole (trimethoprim with sulfamethoxazole) (Polfa). For doxycycline, lincomycin, and sparfloxacin susceptibility testing, French interpretative criteria were applied (6). The microdilution panels were prepared by a single senior technician. The quality control strains *S. pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were included in each run.

RFEL analysis and BOX PCR typing. Typing of pneumococcal strains by RFEL analysis was performed as described by Van Steenbergen et al. (41) and adapted by Hermans et al. (16). Briefly, purified pneumococcal DNA was digested with the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [α-32 P] dATP by using DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. Subsequently, the gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, N.Y.), and exposed for variable lengths of time at room temperature to ECL Hyperfilms (Amersham, Bucks, United Kingdom).

BOX PCR typing was carried out in a single laboratory as described before (40). Briefly, 50 ng of pneumococcal DNA was amplified by PCR (4 min at 94°C [predenaturation]; 40 cycles of 1 min at 94°C; 1 min at 60°C; and 2 min at 74°C; and 2 min at 74°C [extension]), using primer BOX-A (5'ATACTCTTCGAAAATCTCTTCAAAC), which was designed from the primary structure of the BOX repeat motif. The amplified products were separated on a 1.5% agarose gel. Gels were stained with ethidium bromide, after which the banding patterns were evaluated visually. BOX PCR patterns showing a single band difference were defined as nonidentical types (e.g., 1, 2, and 3). Identical banding patterns varying in the intensity of one or more bands were defined as subtypes (e.g., 7, 7.1, and 7.2).

PBP genotyping. Genetic polymorphism of the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* was investigated by restriction fragment length polymorphism (RFLP) analysis. To this end, we amplified the genes by PCR and analyzed the digested DNA products by agarose gel electrophoresis. PCR amplification of the PBP-encoding genes was performed in a 50-μl PCR buffer system containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (wt/vol) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 10 pmol of the individual primers, 0.5 U of DNA polymerase (Eurogentec), and 10 ng of purified chromosomal DNA. Cycling was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, Mass.) and consisted of the following steps: predenaturation at 94°C for 1 min; 30 cycles of 1 min at 94°C; 1 min at 52°C; and 2 min at 72°C; and a final extension at 72°C for 3 min. The primers used to amplify the genes *pbp1a*, *pbp2b*, and *pbp2x* were described previously (5, 7, 24).

The amplification products (5 μl) were digested with the restriction endonuclease *Hin*fI and separated by electrophoresis in 2.5% agarose gels containing 0.5 x Tris-borate-EDTA and 0.1 μg of ethidium bromide per ml (5 mm thick) (Agarose MP; Boehringer Mannheim, Almere, The Netherlands). Gels were run in 0.5 x Tris-borate-EDTA containing 0.1 μg of ethidium bromide per ml at 20 mA for 4 h. Prior to electrophoresis, samples were mixed with a 5 x concentrated layer mix consisting of 50% glycerol in water and 0.8 mg of bromophenol blue per ml. Gels were photographed with a Polaroid MP4 Landcamera and Polaroid 667 films. The different PBP genotypes are represented by a three-number code (e.g., 06-14-43, referring to the RFLP patterns of the genes *pbp1a* [pattern 6], *pbp2b* [pattern 14], and *pbp2x* [pattern 43], respectively.

Computer-assisted analysis of the DNA banding patterns. The RFEL types were analyzed by using the Windows version of the Gelcompar software version 4 (Applied Maths, Kortrijk, Belgium) after imaging the RFEL autoradiograms with the Image Master DTS (Pharmacia Biotech, Uppsala, Sweden). To this end, the DNA fragments in the molecular size range of 160 to 400 bp were explored. The DNA banding patterns were normalized by using pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages (29) and with the Jaccard similarity coefficient applied to peaks (36). Computer-assisted analysis and the methods and algorithms used in this study were according to the instructions of the manufacturer of Gelcompar. A tolerance of 1.5% in band

positions was applied during comparison of the DNA patterns. Identical DNA types were arbitrarily defined as those with RFEL homologies higher than 95%. Cluster codes I to IV refer to clusters of pneumococcal strains displaying RFEL types with homologies higher than 80% that were confirmed to be genetically related by using BOX PCR typing (identical BOX PCR types or subtypes).

RESULTS

The molecular epidemiology of penicillin-nonsusceptible multidrug-resistant pneumococci in Poland was investigated. Thirty-seven patients who suffered mainly from upper and lower respiratory tract infections (n = 20 and 13, respectively) were enrolled in this study. In addition, three patients suffered from otitis media, a brain abscess, and conjunctivitis, respectively. The clinical diagnosis of one patient was unknown. The medical centers to which the patients were referred were located in 16 Polish towns across the country (Fig. 1). The clinical and demographic parameters are listed in Table 1.

The 37 penicillin-nonsusceptible pneumococcal strains were characterized by RFEL analysis and BOX PCR typing. RFEL analysis divided the 37 pneumococcal strains into 16 distinct types (Fig. 2, Table 1). Eight distinct BOX PCR types were observed, representing 14 subtypes (Fig. 3, Table 1). When RFEL and BOX PCR analyses were combined, four genetically distinct clusters of strains (designated I, II, III, and IV) were identified. Within these clusters, genetic relatedness of the strains was demonstrated by both RFEL analysis and BOX PCR (sub)typing. Clusters I, II, III, and IV consisted of 12, 3, 11, and 7 strains, respectively. Each cluster was represented by strains that originated from different centers (Table 1). The remaining four strains did not fulfill the cluster criteria.

Comparison of the Polish RFEL types with 133 genotypes present in the international RFEL data library representing 15 other countries (13, 14) revealed that the genetic clusters II and IV matched the Spanish pandemic clone 23F (RFEL type 15) and the Spanish-French international clone 9V (RFEL type 23), respectively (data not shown). The remaining 14 Polish RFEL types did not match any of the 133 non-Polish types present in the international library. The 37 penicillin-nonsusceptible pneumococci from Poland represented seven distinct serotypes (Table 1). The most predominant serotypes were 23F (n = 15), 6B (n = 12), and 9V (n = 6). Serotypes 6A, 14, and 19A were all observed once in the Polish collection.

Table 1. Microbiological parameters of 37 penicillin-resistant pneumococcal isolates, and clinical and demographic data of the patients.

Patient				Pneumococcal strain							
Age (years)	Sex	Clinical diagnosis	Center[2]	Strain number	Clinical origin	Penicillin MIC (μg/ml)	Resistance profile[3]	Serotype	BOX PCR type	RFEL type[4] (cluster)[5]	PBP genotype
<1	female	URTI[1]	7	39	nose	4.0	PAXCDELT	23F	1	106 (I)	6-2-53
<1	male	URTI	7	40	nose	4.0	PAXCDELT	23F	1	106 (I)	6-1-53
child	-[6]	URTI	6	32	nose	4.0	PAXCDELT	23F	1	106 (I)	6-1-53
1	male	URTI	7	26	nose	4.0	PAXCDELT	23F	1	105 (I)	6-18-53
child	male	URTI	17	29	nose	4.0	PAXCDELT	23F	1	105 (I)	6-18-56
1	male	LRTI[1]	1	6	throath	8.0	PAXCDET	23F	1	105 (I)	16-3-1
<1	male	LRTI	7	9	blood	4.0	PAXCDELT	23F	1	105 (I)	6-18-53
1	male	URTI	14	22	nose	2.0	PAXCDELT	23F	1	145 (I)	6-14-43
6	male	otitis media	4	151	ear	4.0	PA*CDE**	23F	1	44 (I)	6-14-43
4	female	URTI	3	178	nose	8.0	PA*CDE**	23F	1	44 (I)	6-14-1
4	male	URTI	4	233	throat	8.0	PA*CDE**	23F	1	45 (I)	6-14-1
5	female	URTI	4	234	throat	8.0	PA*CDE**	23F	1	45 (I)	6-14-1
adult	female	LRTI	2	4	sputum	2.0	PAXCDET	23F	2	15 (II)	1-1-1
13	female	LRTI	18	16	BAL[1]	2.0	PAXDET	23F	2.1	15 (II)	1-1-1
3	male	URTI	16	33	throath	2.0	PAXCDET	23F	2	15 (II)	1-18-1
57	male	brain abscess	12	8	CSF[1]	0.5	PCDELT	19A	3	104	7-17-52
55	male	LRTI	10	2	BAL	1.0	PCT	6A	4	97	2-5-51
-	-	-	8	31	-	1.0	PD	rough	5	148	0[7]-2-54
3	female	URTI	7	36	nose	1.0	PCDELT	6B	6	147	0-1-54
14	male	LRTI	9	12	sputum	0.12	PCDELT	6B	7	98 (III)	2-2-9
7	male	LRTI	9	13	sputum	0.12	PCDELT	6B	7	98 (III)	2-2-9
3	female	LRTI	9	14	blood	0.12	PCDELT	6B	7.1	98 (III)	2-2-9
1	male	URTI	13	35	nose	0.12	PCDELT	6B	7.2	98 (III)	2-18-9
6	male	URTI	16	34	nose	0.12	PCDELT	6B	7.1	98 (III)	2-18-9

Table 1. Microbiological parameters of 37 penicillin-resistant pneumococcal isolates, and clinical and demographic data of the patients (cont'd).

Patient				Pneumococcal strain							
Age (years)	Sex	Clinical diagnosis	Center[2]	Strain number	Clinical origin	Penicillin MIC (μg/ml)	Resistance profile[3]	Serotype	BOX PCR type	RFEL type[4] (cluster)[5]	PBP genotype
adult	-	LRTI	11	17	sputum	0.12	PCDELT	6B	7.3	102 (III)	2-2-9
2	male	URTI	14	23	nose	0.12	PDE	6B	7.3	102 (III)	2-2-9
child	male	conjunctivitis	2	30	eye	0.12	PDEL	6B	7.1	102 (III)	2-2-9
12	male	LRTI	9	15	female	0.12	PDEL	6B	7.2	101 (III)	2-2-9
<1	-	LRTI	2	5	throath	0.12	PDEL	6B	7.3	99 (III)	2-2-9
5	male	URTI	17	27	nose	0.12	PCDET	6B	7.4	99 (III)	2-2-9
child	female	URTI	7	24	nose	1.0	PACDT	9V	8	23 (IV)	1-1-1
adult	male	URTI	17	38	nose	1.0	PAXCDET	9V	8.1	23 (IV)	1-2-1
adult	female	LRTI	6	1	pleural fluid	1.0	PAXCDET	9V	8.1	23 (IV)	1-1-1
child	male	URTI	5	10	nose	1.0	PAXCDET	9V	8	23 (IV)	1-5-1
adult	-	LRTI	15	11	sputum	2.0	PAXCDELT	14	8	23 (IV)	1-5-1
child	female	URTI	3	37	throath	0.5	PXCDT	9V	8.1	23 (IV)	1-2-1
10	female	URTI	19	181	throath	1.0	PA*C**	9V	8.1	140 (IV)	1-1-1

[1]URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; BAL, broncheo-alveolar lavage; CSF, cerebro-spinal fluid.
[2]Codes refer to geographical areas depicted in Figure 1.
[3]P, penicillin G; A, amoxicillin; X, cefotaxime, C, chloramphenicol; D, doxycycline; E, erythromycin; L, lincomycin; T, trimethoprim- sulfamethoxazole (cotrimoxazole). *Susceptibility to cefotaxime, lincomycin and cotrimoxazole was not tested.
[4]The arbitrary cut-off value for an identical RFEL type is 95%; RFEL codes exclusively refer to the genetic distances calculated using the entire collection of 147 pneumococcal RFEL types, in which the Polish genotypes were included (see materials and methods section) (13, 14).
[5]Roman numbers refer to cluster codes depicted in Figure 2.
6- ,data not available.
[7]Unknown PBP genotype.

Strain 31 displayed a rough phenotype and could not be serotyped. Serotypes 23F, 6B, and 9V included 6, 5, and 2 RFEL types, respectively, whereas the remaining three serotypes (6A, 14, and 19A) were restricted to single RFEL types. The clusters I, II (pandemic clone 23F), and III displayed the serotypes 23F, 23F, and 6B, respectively. Genetic cluster IV (pandemic clone 9V) harbored both serotypes 9V (n = 6) and 14 (n = 1), indicating horizontal transfer of capsular genes within this cluster.

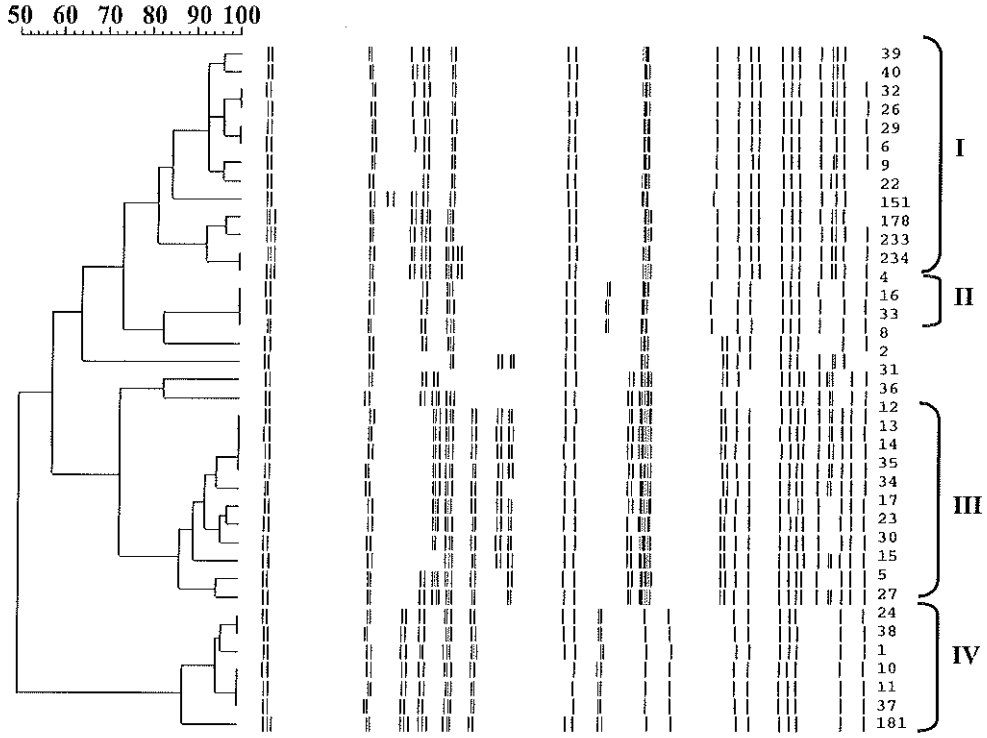

Fig. 2. Genetic relatedness of 37 Polish penicillin-nonsusceptible pneumococcal strains based on the RFEL banding patterns of the isolates. The RFEL fingerprints, their relatedness (dendrogram), strain numbers, and genetic cluster codes (Roman numerals) are depicted. Cluster codes I to IV refer to clusters of pneumococcal strains displaying RFEL types with homologies higher than 80% that are confirmed to be genetically related by BOX PCR typing (identical BOX PCR types or subtypes).

The penicillin MICs of the pneumococcal strains varied from 0.12 to 8.0 μg/ml. Within the genetic clusters, various resistance profiles were observed, ranging from lack of susceptibility to penicillin and doxycycline only to multidrug resistance against penicillin, cefotaxime, cotrimoxazole, doxycycline, chloramphenicol, erythromycin, and lincomycin. Genetic cluster I had the highest penicillin MICs (up to 8 μg/ml), as well as the greatest multidrug resistance

(Table 1). The lowest penicillin MICs were observed for isolates of genetic cluster III. The resistance patterns of these strains demonstrated the irrelative susceptibilities to the antimicrobial agents tested. Nevertheless, all members of cluster III were multidrug-resistant, i.e., resistant to three or more antibiotics. None of the isolates were resistant to vancomycin, rifampin, and fluoroquinolones.

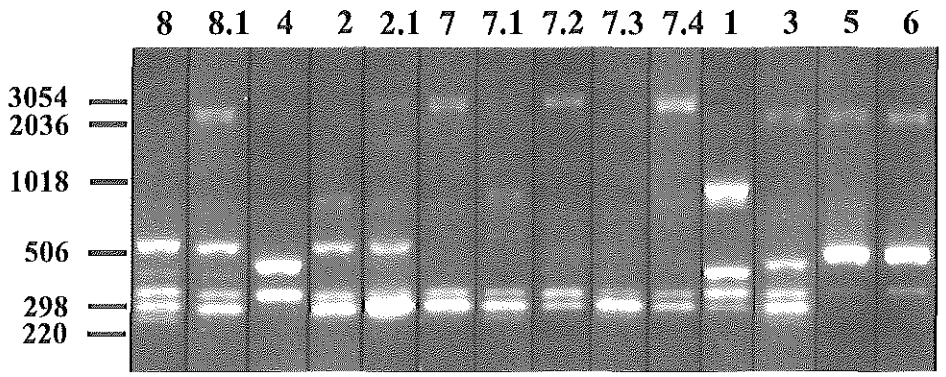

Fig. 3. DNA fingerprint patterns of the 14 distinct BOX PCR (sub)types represented by the 37 Polish penicillin-nonsusceptible pneumococcal strains. Lane numbers indicate BOX PCR type codes. Numbers at the left indicate the sizes of standard DNA fragments in base pairs.

The penicillin resistance profiles of the pneumococcal strains were investigated in further detail by PBP genotyping. To this end, the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* were subjected to RFLP analysis (Fig. 4). The genetic clusters II (pandemic clone 23F) and IV (pandemic clone 9V) included two and three distinct PBP genotypes, respectively (Table 1). The polymorphism was restricted to *pbp2b*. Similar results were observed for cluster III: there were two distinct PBP genotypes that differed only in the *pbp2b* gene. For cluster I, seven distinct PBP genotypes were observed; in contrast to the case for the other three genetic clusters, genetic polymorphism was demonstrated in *pbp1a* (two types), *pbp2b* (five types), and *pbp2x* (four types). Except for clusters II and IV, representing pandemic clones 23F and 9V, respectively, no overlap was observed between the PBP genotypes of the four clusters. Similarly, the four genotypes that were observed only once in the Polish collection also displayed unique PBP genotypes. The polymorphism of the PBP-encoding genes within clusters II and IV was remarkable, as the majority of the pandemic 23F and 9V isolates that are now present in the international data library (51 23F strains from 10 countries [96%] and 31 9V strains from 5 countries [97%]) display PBP genotype 1-1-1 (13, 14).

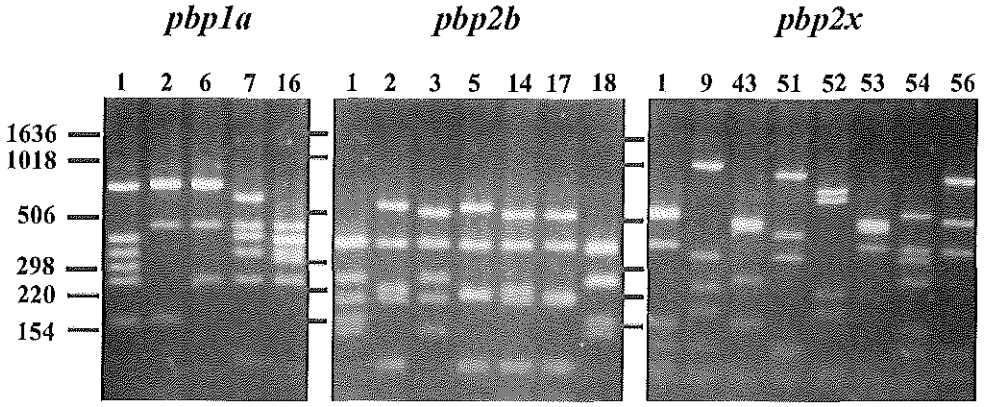

Fig. 4. DNA fingerprint patterns of the *pbp1a* ($n = 5$), *pbp2b* ($n = 7$), and *pbp2x* ($n = 8$) genotypes represented by the 37 Polish penicillin-nonsusceptible pneumococcal strains. Lane numbers indicate PBP genotype codes. Numbers at the left indicate the sizes of standard DNA fragments in base pairs.

Comparison of the PBP genotypes of the individual genes *pbp1a*, *pbp2b*, and *pbp2x* between the Polish penicillin-nonsusceptible strains and 185 Dutch penicillin-susceptible pneumococcal meningitis isolates (15, 32) revealed the exclusive presence of penicillin-susceptible *pbp1a* and *pbp2b* genotypes among the Polish clusters I, III, and IV. Two strains of cluster IV (pandemic clone 9V; strains 37 and 38 with penicillin MICs of 0.5 and 1.0 μg/ml, respectively) displayed the predominant penicillin-susceptible *pbp2b* genotype 2 (Fig. 4, Table 1). In addition, the majority of the strains belonging to cluster III displayed the predominant penicillin-susceptible *pbp1a-pbp2b* genotype 2-2 (penicillin MIC of 0.12 μg/ml). Finally, the majority of cluster I displayed the penicillin-susceptible *pbp1a* genotype 6 (penicillin MICs ranging from 2.0 to 8.0 μg/ml); this genotype was observed only once in the collection of 185 Dutch penicillin-susceptible pneumococci.

Among the *pbp1a*, *pbp2b*, and *pbp2x* genotypes that are restricted to penicillin-nonsusceptible pneumococci (13, 14), the presence of *pbp1a* genotype 1 was observed among clusters II (clone 23F) and IV (clone 9V) (Table 1). In addition, the *pbp2b* genotypes 1 and 18 and the *pbp2x* genotype 1 were observed in three genetic clusters. These data indicate horizontal exchange of penicillin resistance genes between pneumococcal strains of the different Polish clusters.

DISCUSSION

Penicillin resistance among pneumococcal isolates has rapidly emerged in Poland during the last decade. The percentage of penicillin-nonsusceptible strains has increased from 0 to 3% in 1990 to 1993 (1, 19) to 14.3% in 1996 (39) and 14.4% in 1997 (38). In order to identify the nature of the increase in the prevalence of penicillin-nonsusceptible pneumococci in Poland, a molecular epidemiological study was conducted. The emergence of four genetically distinct penicillin-nonsusceptible multidrug-resistant pneumococcal clusters was clearly demonstrated. Clusters II and IV represented the genetic clones 23F (24) and 9V (11), respectively, which have recently emerged all over the world (13, 14). In addition, the two other clusters, I and III, predominated in the collection of penicillin-nonsusceptible pneumococci from Poland. Since these clusters did not match any of the 133 RFEL types of penicillin-nonsusceptible pneumococci collected in 15 other countries and present in the international data library of RFEL types (13, 14), their Polish origin is most likely.

Various researchers have reported horizontal transfer of capsular genes (2, 13–15). The high frequency of capsular transfer may have consequences for the outcome of current vaccine strategies, which focus on the use of a restricted number of distinct capsular polysaccharides. The use of multivalent conjugate vaccines may shift the capsular distribution towards capsular types that are not present in these vaccines. Such a shift might be enhanced by the frequent horizontal exchange of capsular genes. Genetic cluster IV (pandemic clone 9V) harbored both serotypes 9V ($n = 6$) and 14 ($n = 1$). These serotypes are known to occur in this international clone (13,14). The Polish strains that were genetically related to the pandemic clone 23F (cluster II), which harbors serotypes 23F, 19F, 14, 9N, 19A, and 3 (13, 14, 27), displayed only the ancestral serotype 23F.

Within the four genetic clusters, RFLP of the PBP-encoding genes was often observed, in particular in *pbp2b*. This finding indicates frequent horizontal transfer of the penicillin resistance genes. PBP genotype 01-01-01 was observed in clusters II (pandemic clone 23F) and IV (pandemic clone 9V). In a recent study by Hermans and coworkers (13), the vast majority of isolates of the pandemic clones 23F and 9V also displayed the PBP genotype 01-01-01. In that study, PBP genotype 01-01-01 was displayed by 41% of the strains collected in 15 other countries and represented 20 of the 133 distinct RFEL types. In addition, genetic polymorphism in the penicillin resistance genes of the pandemic clones 9V and 23F was rarely observed. However, in five of the 10 Polish strains that were related to the pandemic clones 9V and 23F, differences in the PBP genotypes were observed, suggesting that in

Poland horizontal transfer of penicillin resistance genes, in particular *pbp2b*, occurs more frequently in these clones than in those present in the other countries. The *pbp2b* genotypes 18 (observed only in Poland so far) and 1 and the *pbp2x* genotype 1 were observed in three of the four genetic clusters. These data suggest that these PBP genotypes have spread in the Polish population of penicillin-nonsusceptible pneumococci by horizontal gene transfer.

The strains belonging to cluster III invariably displayed low penicillin MICs of 0.12 μg/ml. In addition, the majority of these strains displayed the penicillin-susceptible *pbp1a-pbp2b* genotype 2-2. Although the predictive value of PBP genotyping for pneumococcal penicillin resistance can be debated for methodological reasons, this observation is in concordance with data from Smith and coworkers (35), who have demonstrated that in pneumococci with low penicillin MICs, *pbp2b* is usually not affected by mutations.

In summary, penicillin resistance has rapidly emerged in Poland over the last decade. Although the pandemic clones 9V and 23F are present in this country, the nationwide emergence of two novel multidrug-resistant pneumococcal clones that are presumably of Polish origin has clearly contributed to the increase in the prevalence of penicillin-nonsusceptible pneumococci in Poland.

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Chapter 4

Nasopharyngeal carriage of penicillin-resistant *Streptococcus pneumoniae* among children with acute respiratory tract infections in Thailand: a molecular epidemiological survey

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SUMMARY

The prevalence of penicillin-resistant *Streptococcus pneumoniae* in Thailand has dramatically increased over the last decade. During a national survey, which was conducted from 1992 to 1994, 37.2% of the pneumococci isolated from the nasopharynges of children with acute respiratory tract infections were penicillin-resistant (MIC, ≥0.1 μg/ml). In order to investigate the prevalence and clonal relatedness of nasopharyngeal carriage of penicillin-resistant *S. pneumoniae* in Thailand, a molecular epidemiological survey was undertaken. To this end, 53 penicillin-resistant pneumococcal isolates from children who suffered from acute respiratory tract infections and who originated from five distinct regions of the country were characterized in detail. DNA fingerprint analysis demonstrated 13 clusters, i.e., genotypes shared by two or more strains, and 14 unique genotypes. The cluster size varied from 2 (nine clusters) to 11 strains (one cluster). Six of the 13 restriction fragment end labeling clusters consisted of two or more distinct serotypes, indicating frequent horizontal transfer of capsular genes. Geographical distribution of the genotypes among the five regions of Thailand demonstrated that only four genetic clusters were restricted to single areas of the country, whereas the other nine clusters represented isolates collected in two or more districts. These observations demonstrate that the majority of the genetic clusters are spread throughout the country. The most predominant genetic cluster, representing 21% of the isolates, was identical to the Spanish pandemic clone 23F. In addition, the second largest cluster matched the Spanish-French international clone 9V. These data indicate that the genetic clones 23F and 9V, which are widely spread throughout the world, are the most predominant multidrug-resistant pneumococcal clones in Thailand. Therefore, we conclude that these pandemic clones are primarily responsible for the increase in the prevalence of pneumococcal penicillin resistance in Thailand.

INTRODUCTION

Streptococcus pneumoniae is one of the leading bacterial pathogens causing illness and death among young children, the elderly, and persons with underlying medical conditions, such as immunocompromised patients (5). Pneumococcal infection is usually preceded by colonization of the human nasopharynx. This is an important step toward infection. Consequently, pneumococcal colonization is an important risk factor for developing disease.

For instance, young children who are frequently colonized with pneumococci more often develop acute otitis media than do children who are not or less frequently colonized (12, 19, 33, 43).

The prevalence of penicillin resistance among pneumococci, both high-level resistance (MIC of >1 μg/ml) and intermediate-level resistance (MIC of 0.1 to 1 μg/ml), is alarmingly increasing worldwide (4, 8, 14, 21–24, 42). International spread of a restricted number of multiresistant pneumococcal clones has significantly contributed to this increase. Soares and co-workers have documented the spread of a multiresistant clone of serotype 6B from Spain to Iceland in the late 1980s (35). This has resulted in an epidemic of this clone, which was isolated with a frequency of up to 12% as early as 1992 (22). In 1991, Munoz and colleagues reported evidence for the intercontinental spread of a multiresistant clone of *S. pneumoniae* serotype 23F from Spain to the United States (26). This clone has subsequently disseminated throughout the United States (25). Finally, Gasc and colleagues in 1995 reported the spread of a multiresistant pneumococcal clone of serogroup 9 from Spain to France (13). The international clones 23F and 9V have been identified in many countries all over the world (16, 30). Besides the international spread of the clones 6B, 23F, and 9V, novel penicillin-resistant and multiresistant clones in the former Czechoslovakia, Spain, Japan, and South Africa that tend to spread in an epidemic manner within these countries have been reported (6, 30, 31, 34, 41).

In Thailand, a national survey conducted from 1992 to 1994 demonstrated a prevalence of penicillin-resistant pneumococci (MIC of ≥0.1 μg/ml) as high as 37.2% (1, 29, 37). This figure was much higher than those in the surveys in 1978 (6.7%) (36) and in 1987 (10.6%) (20). In order to identify the nature of the increase in the prevalence of penicillin-resistant pneumococci in Thailand, a molecular epidemiological study was undertaken. To this end, strains isolated from the nasopharynges of 53 pediatric patients, who suffered from acute respiratory tract infections and who originated from different regions across the country, were examined. The pneumococcal isolates were characterized by restriction fragment end labeling (RFEL) analysis, BOX PCR typing, penicillin-binding protein (PBP) genotyping, serotyping, and drug susceptibility testing.

MATERIALS AND METHODS

Bacterial strains. In the survey conducted in 1978, susceptibility testing was performed with 446 isolates. One hundred seventeen isolates were collected in Bangkok from patients who were admitted to three university hospitals (Siriraj Hospital, Chulalongkorn Hospital, and Ramathibodi Hospital) and a private hospital (Seventh Day Adventist Hospital). These pneumococci were collected mainly from blood (26%), throat swab (23%), sputum (17%), cerebrospinal fluid (10%), and pus (7%) samples. In addition, 329 isolates were obtained from healthy carriers (ranging from 3 to 45 years of age) in Bangkok (80%) and Samutprakarn (20%), a province near Bangkok. The survey conducted in 1986 comprised 95 pneumococcal isolates from patients who were admitted to the Siriraj University Hospital in Bangkok. The isolates were collected mainly from blood (57%), sputum (20%), pus (12%), cerebrospinal fluid (4%), and pleural fluid (4%) samples.

During a national survey of antimicrobial resistance in Thailand from 1992 to 1994 (1, 29, 37), nasopharyngeal swabs were taken from 1,783 children under the age of 5 years with acute respiratory infections. The children were hospitalized in six hospitals representing five regions of Thailand (Fig. 1): Children's Hospital Bangkok (central region), Cholburi Hospital (eastern region), Khonkaen Hospital and Maharaj Nakorn Ratchasima Hospital (northeastern region), Phra Buddha Chinaraj Hospital (northern region), and Hatyai Hospital (southern region). *S. pneumoniae* species identification was determined by optochin sensitivity and bile solubility testing (27). In this survey, 615 pneumococci were isolated (isolation rate, 34.5%). All 615 strains were referred to our laboratory, and 530 strains were viable at the time of arrival. The antibiotic resistance profile of these 530 strains was determined (see below). The collection represented 197 penicillin-resistant pneumococcal isolates (MIC of ≥0.1 μg/ml). Twenty-six of 36 high-level penicillin-resistant pneumococcal isolates (MIC of >1.0 μg/ml) and 27 of 161 intermediate-level-resistant pneumococci (MIC of 0.1 to 1 μg/ml) were randomly selected for this study (Table 1).

The MICs for the pneumococcal strains were determined by agar dilution. The MIC was defined as the lowest concentration of the antimicrobial agent preventing visible growth. To this end, serial ^{2}log concentrations of antibiotics were prepared in IsoSensitest agar (Oxoid, Unipath Ltd., Basingstoke, United Kingdom), supplemented with 5% horse blood. The pneumococcal isolates were removed from storage at -70°C and subcultured at 37°C on Columbia agar (Oxoid) supplemented with 5% sheep blood with 5% CO_2. Bacterial

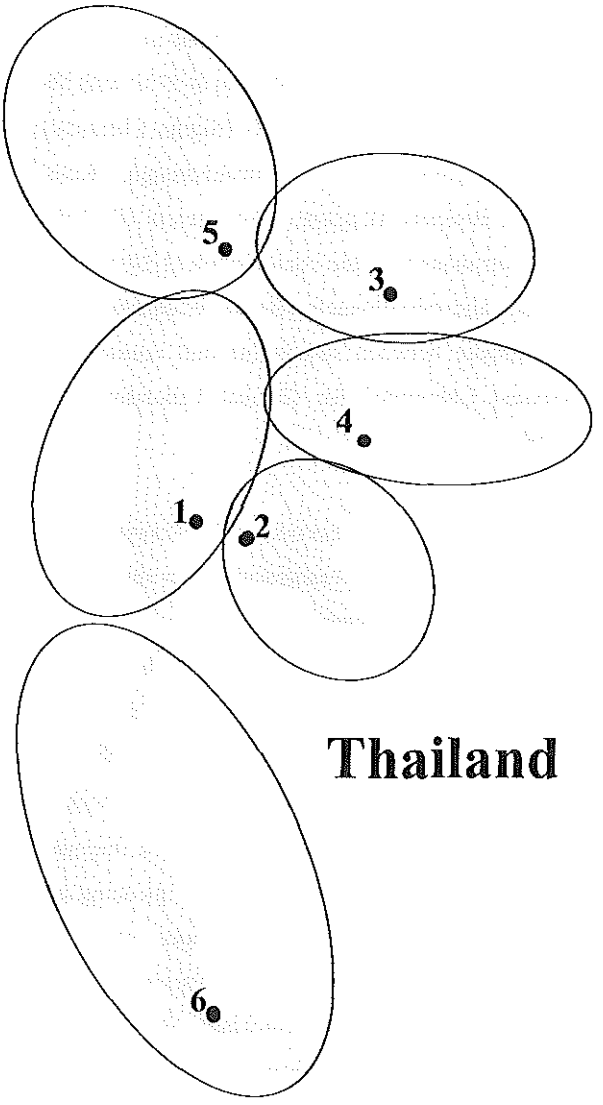

Fig. 1. Geographical map of Thailand. The medical centers collaborating in this study are indicated by numbers: 1, Children's Hospital Bangkok (central region); 2, Cholburi Hospital (eastern region); 3, Khonkaen Hospital (northeastern region); 4, Maharaj Nakron Ratchasima Hospital (northeastern region); 5, Phra Buddha Chinaraj Hospital (northern region); 6, Hatyai Hospital (southern region). The geographical regions serviced by the medical centers are depicted by open circles.

suspensions were prepared in 0.9% NaCl from 24-h agar cultures and adjusted to a McFarland turbidity standard of 0.5. Suspensions were further diluted (1:10) in saline. The inocula were applied on the test plates with a multipoint inoculator, resulting in about 10^4 CFU per spot. MICs were read after 24 h of incubation at 37°C with 5% CO_2.

The antimicrobial agents tested were penicillin G (Sigma Chemical Co., St. Louis, Mo.), erythromycin (Abbott Laboratories, Ltd., Queenborough, Kent, United Kingdom), doxycycline (Pfizer S. A., Brussels, Belgium), vancomycin (Eli Lilly & Co., Indianapolis, Ind.), rifampin (Sigma), cotrimoxazole, the combination (1:19) of trimethoprim (Sigma) and sulfamethoxazole (Sigma), and ciprofloxacin (Bayer, Wuppertal, Germany). Breakpoints of the antibiotics to discriminate between susceptible and nonsusceptible strains were used according to the National Committee for Clinical Laboratory Standards guidelines for susceptibility testing (28).

Bacterial typing. (i) Serotyping. Pneumococci were serotyped on the basis of capsular swelling (quellung reaction) observed microscopically after suspension in antisera prepared at Statens Serum Institut, Copenhagen, Denmark (11).

(ii) RFEL analysis. Typing of pneumococcal strains by RFEL was performed as described by Van Steenbergen et al. (40) and adapted by Hermans et al. (18). Briefly, purified pneumococcal DNA was digested by the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [α-32 P] dATP with *Taq* DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. Subsequently, the gel was transferred onto filter paper, vacuum dried (Haake Buchler Instruments Inc., Saddlebrook, N.Y.), and exposed for varying periods at room temperature to ECL Hyperfilms (Amersham, Little Chalfont, Buckinghamshire, United Kingdom).

The RFEL types were analyzed with the Windows version of the Gelcompar software version 4 (Applied Maths, Kortrijk, Belgium) after the RFEL autoradiograms were scanned with Image Master DTS (Pharmacia Biotech, Uppsala, Sweden). For this purpose, the DNA fragments in the molecular size range of 160 to 400 bp were examined. The fingerprints were normalized with pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the fingerprints was performed by the unweighted pair group method using arithmetic averages and by using the Jaccard similarity coefficient applied to peaks. Computer-assisted analysis, methods, and algorithms used in this study were carried out or

used according to the instructions of the manufacturer of Gelcompar. A tolerance of 1.5% in band positions was applied during comparison of the fingerprint patterns. Identical DNA types were arbitrarily defined as RFEL homologies higher than 95%. A genetic cluster was defined as a genotype (RFEL or PBP type) that was shared by two or more pneumococcal strains. The degree of genetic clustering was defined as the percentage of strains displaying genotypes (RFEL or PBP types) that were observed twice or more.

(iii) BOX PCR typing. BOX PCR typing was carried out as described before (39). Briefly, 50 ng of pneumococcal DNA was amplified by PCR (4 min at 94°C [predenaturation]; 40 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 74°C; and 2 min at 74°C [extension]), with primer BOX-A, designed on the primary structure of the BOX repeat motif. The amplified products were separated on a 1.5% agarose gel. Gels were stained with ethidium bromide, after which the banding patterns were evaluated visually. BOX PCR patterns showing a single band difference were defined as nonidentical types. BOX PCR was used as a confirmatory method to verify genetic clustering observed by RFEL analysis.

(iv) PBP genotyping. Genetic polymorphism of the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* was investigated by restriction fragment length polymorphism analysis. To this end, we amplified the genes by PCR and analyzed the digested DNA products by agarose gel electrophoresis. PCR amplification of the PBP-encoding genes was performed in a 50-μl PCR buffer system containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (wt/vol) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 10 pmol of the individual primers, 0.5 U of Goldstar *Taq* DNA polymerase (Eurogentec, Liège, Belgium), and 10 ng of purified chromosomal DNA. Cycling was performed in a PTC-100 programmable thermal controller (MJ Research, Watertown, Mass.) and consisted of the following steps: predenaturation at 94°C for 1 min; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C; and final extension at 72°C for 3 min. The primers used to amplify the genes *pbp1a*, *pbp2b*, and *pbp2x* were described previously (7, 10, 26). The amplification products (5 μl) were digested by restriction endonuclease *Hin*fI and separated by electrophoresis in 2.5% agarose gels (5 mm thick; Agarose MP; Boehringer Mannheim, Almere, The Netherlands) containing 0.5 x Tris-borate-EDTA and 0.1 μg of ethidium bromide per ml. Gels were run in 0.5 x Tris-borate-EDTA containing 0.1 μg of ethidium bromide per ml at a constant current of 20 mA for 4 h. Prior to electrophoresis, samples were mixed with a 5 x concentrated layer mix consisting of 50% glycerol in water and 0.8 mg of bromophenol blue per ml. Gels were photographed with a Polaroid MP4 Landcamera and Polaroid 667 films. The different PBP

genotypes are represented by a three-number code (e.g., 01-06-13), referring to the restriction fragment length polymorphism patterns of the genes *pbp1a* (pattern 1), *pbp2b* (pattern 6), and *pbp2x* (pattern 13), respectively.

RESULTS

From 1992 to 1994, the prevalence of penicillin-resistant pneumococci in Thailand was determined during a national antimicrobial resistance survey. This study showed a countrywide penicillin resistance rate (MIC of ≥0.1 μg/ml) of 37.2%; 6.8% of the pneumococcal isolates showed high-level resistance to penicillin, whereas 30.4% of the isolates were intermediate-level resistant (Table 1). The resistance rates varied from region to region, ranging from 17.1% in Hatyai Hospital (southern region) to 59.2% in Phra Buddha Chinaraj Hospital (northern region). The latter region had the highest prevalence of high-level pneumococcal penicillin resistance (28.6%), followed by the Children's Hospital Bangkok (central region; 6.9%). Twenty-three of the 26 randomly selected high-level penicillin-resistant isolates and 14 of the 27 intermediate-level penicillin-resistant isolates were resistant to more than three antibiotics with resistance profile penicillin–trimethoprim-sulfamethoxazole–doxycycline–erythromycin–chloramphenicol, penicillin–trimethoprim-sulfamethoxazole–doxycycline–erythromycin, or penicillin–trimethoprim-sulfamethoxazole–doxycycline–chloramphenicol. None of the isolates were resistant to vancomycin, rifampin, or ciprofloxacin.

All 53 penicillin-resistant pneumococcal isolates were analyzed by RFEL. This DNA fingerprint method divided the strains into 27 distinct types (Fig. 2, Table 1). These RFEL types represented 13 clusters, i.e., types shared by two or more strains, and 14 unique types. Thirty-nine strains shared RFEL types with at least one other strain (74%). The cluster size varied from two (nine clusters) to 11 strains (one cluster). In addition, two clusters of three strains and one cluster of four strains were observed. For 10 RFEL clusters, genetic identity was confirmed by BOX PCR typing. Within the remaining three RFEL clusters, strong genetic relatedness was observed by BOX PCR typing (data not shown). Although the degree of genetic clustering, i.e., the percentage of strains sharing their RFEL types with one or more other strains, was comparable between the groups of high-level and intermediate-level penicillin-resistant strains (high-level, 50%; intermediate-level, 48%), a reduced number of RFEL

types was observed among the high-level penicillin-resistant isolates (15 high-level penicillin resistance RFEL types and 20 intermediate-level penicillin resistance RFEL types [Fig. 2]).

Table 1. Prevalence of penicillin-resistant pneumococci among 1,783 children with acute respiratory tract infections[a].

Hospital	No. of isolates	HPRP			IPRP			% HPRP plus IPRP
		No.	%	No. of randomly selected for this study	No.	%	No. of randomly selected for this study	
B	216	15	6.9	7	82	38.0	16	44.9
C	44	2	4.5	2	16	36.4	4	40.9
K	53	1	1.9	1	10	18.9	3	20.8
N	86	3	3.5	2	25	29.1	2	32.6
P	49	14	28.6	13	15	30.6	2	59.2
S	82	1	1.2	1	13	15.9	0	17.1
Total	530	36	6.8	26	161	30.4	27	37.2

[a]Data were collected during the National Pneumococcal Antimicrobial Resistance Surveillance between 1992 and 1994. From this collection, 26 high-level and 27 intermediate-level penicillin-resistant isolates were randomly selected for detailed molecular epidemiological analysis. Abbreviations: B, Children's Hospital Bangkok; C, Cholburi Hospital; K, Khonkaen Hospital; N, Maharaj Nakorn Ratchasima Hospital; P, Phra Buddha Chinaraj Hospital; S, Hatyai Hospital; HPRP, high-level penicillin-resistant pneumococci; IPRP, intermediate-level penicillin-resistant pneumococci.

Comparison of the 27 Thai RFEL types with 107 additional types present in the international RFEL data library and representing 15 other countries (15, 16) revealed that the most predominant cluster matched the Spanish pandemic clone 23F (RFEL type 15), whereas RFEL cluster 23, representing four 9V strains, was identical to the Spanish-French international clone 9V, respectively. Strain P390, displaying RFEL type 17, was genetically identical to a penicillin-resistant pneumococcal strain isolated in The Netherlands. In addition, the remaining 24 Thai RFEL types did not match any of the 107 types present in the international library.

The 53 pneumococcal isolates comprised eight distinct serotypes. In addition, one strain displayed a nontypeable capsular type. The capsular types of the high-level resistant isolates were restricted to 6A, 6B, 9V, 19F, and 23F. The intermediate-level penicillin-resistant pneumococci harbored the serotypes 6A, 6B, 9V, 14, 15B, 15C, 19F, and 23F (Table 2). Six of the 13 RFEL clusters consisted of two or more distinct serotypes. The most predominant RFEL cluster, 15, consisting of 11 strains, harbored three distinct serotypes, 23F, 19F, and 14 (Table 2). Based on the serotype distribution of the 53 penicillin-resistant pneumococci, 89% of the strains display capsular types that are covered by the 7-valent conjugate vaccine in which the serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F are represented.

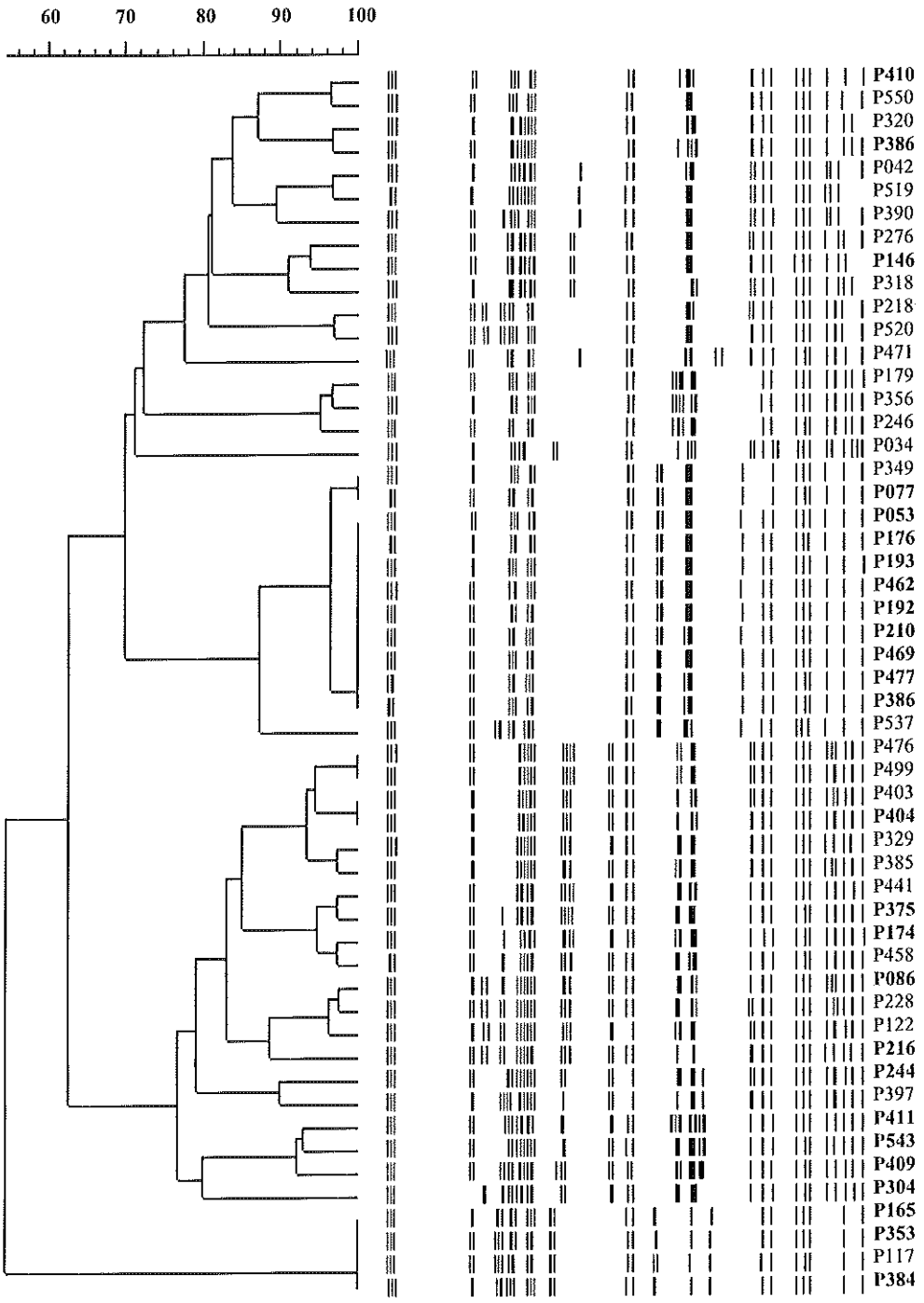

Fig. 2. Genetic relatedness of 53 penicillin-resistant pneumococcal isolates from Thailand. The RFEL fingerprint patterns of the strains (designated by numbers) and their genetic relatedness (dendrograph) are depicted (for details, see text). High-level penicillin-resistant isolates are marked in boldface.

The 53 penicillin-resistant pneumococcal strains were collected in six hospitals representing five regions of the country: Children's Hospital Bangkok (central region; $n = 23$), Cholburi Hospital (eastern region; $n = 6$), Khonkaen Hospital and Maharaj Nakorn Ratchasima Hospital (northeastern region; $n = 4$ and 4, respectively), Phra Buddha Chinaraj Hospital (northern region; $n = 15$), and Hatyai Hospital (southern region; $n = 1$). The RFEL clusters 66 ($n = 2$), 67 ($n = 2$), 68 ($n = 2$), and 70 ($n = 2$) were observed in single regions of the country, whereas the other nine clusters represented isolates collected in two or more regions (Table 2).

All strains were analyzed by PBP genotyping. Sixteen distinct PBP genotypes were observed. The representative types are summarized in Fig. 3. The PBP types 01-01-01 and 01-10-13 were the most predominant penicillin resistance types and represented 16 and 10 strains, respectively. PBP type 01-01-01 was observed in four distinct RFEL types representing five distinct regions, whereas PBP type 01-10-13 was displayed by seven different RFEL types representing three distinct regions. Fourteen PBP type 01-01-01 strains (88%) and four PBP type 01-10-13 strains (40%) were high-level penicillin resistant (Table 2). The PBP types 01-01-01 and 01-10-13 were observed in 11 and 4 of the 16 countries that are currently represented in the international database (15). Twelve of the 16 PBP genotypes were Thailand specific, as they were not observed so far in any of the other countries representing 80 distinct PBP genotypes (15). The *pbp2x* type 13 was observed in 23 strains isolated in four distinct regions of the country and represented 17 RFEL types (Table 2). The vast majority of the 23 *pbp2x* type 13 strains displayed serotype 6B ($n = 20$). Comparison of the PBP genotypes of the individual genes *pbp1a*, *pbp2b*, and *pbp2x* between the 53 Thai penicillin-resistant strains and 185 Dutch and 10 Thai penicillin-susceptible pneumococcal isolates (17, 32) did not demonstrate any overlap (data not shown).

DISCUSSION

In Thailand, a national survey conducted from 1992 to 1994 demonstrated a prevalence of penicillin-resistant pneumococci of 37.2% (1, 29, 37). This figure was much higher than that demonstrated in the surveys in 1978 (6.7%) (36) and in 1987 (10.6%) (20) (details of the 1978 and 1987 surveys are available in Materials and Methods). In order to identify the nature of the increase in the prevalence of penicillin-resistant pneumococci in Thailand, a molecular epidemiological study in which 53 penicillin-resistant pneumococcal isolates collected from 1992 to 1994 were analyzed in detail was conducted.

Table 2. Microbiological parameters of 53 penicillin-resistant pneumococcal isolates and demographic data of the patients.

Strain	Hospital[a]	RFEL	PBP genotype			Serotype	Penicillin MIC[d]	Resistance profile[b]
		type	1a	2b	2x			
P410	P	39	01	06	13	6B	2.000	PTDEC
P550	C	39	02	04	41	15B	0.500	P
P320	B	38	16	01	13	6B	1.000	PTDE
P386	P	38	01	01	01	23F	2.000	PTDEC
P042	P	42	02	02	34	19F	0.125	PTDEC
P519	B	42	11	02	30	19F	0.250	PDE
P390	B	17	01	10	13	6B	1.000	PTDEC
P276	C	40	14	02	38	14	0.250	PDC
P146	C	41	16	01	13	6B	2.000	PTDE
P318	B	92	15	02	39	14	0.125	PT
P218	N	35	02	04	13	15C	0.250	PC
P520	B	35	02	04	41	15B	0.250	PTDE
P471	B	50	00	12	01	23F	1.000	PTDE
P179	K	51	02	05	36	14	0.250	P
P356	B	51	01	10	13	6B	1.000	PTC
P246	P	51	02	05	36	14	0.250	P
P034	C	58	12	09	01	NT[c]	0.500	PTDC
P349	N	15	14	02	38	14	0.125	PDC
P077	P	15	01	01	01	23F	2.000	PTDEC
P053	P	15	01	01	01	23F	2.000	PTDEC
P176	B	15	01	01	01	19F	2.000	PTDEC
P193	S	15	01	01	01	23F	2.000	PTDEC
P462	B	15	01	01	01	19F	2.000	PTDEC
P192	P	15	01	01	01	23F	2.000	PTDEC
P210	P	15	01	01	01	23F	2.000	PTDEC
P469	B	15	01	01	01	19F	2.000	PTDC
P477	B	15	01	01	01	23F	2.000	PTDEC
P386	P	15	01	01	01	23F	2.000	PTDEC
P537	B	48	01	01	01	9V	1.000	PTDE
P476	B	68	01	03	13	6B	1.000	PTDEC
P499	B	68	01	03	13	6B	1.000	PTDEC
P403	C	70	01	10	13	6A	1.000	PTDEC
P404	C	70	01	10	13	6A	2.000	PTDEC
P329	B	69	17	03	40	19F	0.500	PDE
P385	K	69	02	06	36	14	0.125	PT
P441	B	67	01	03	13	6B	1.000	PTDEC
P375	B	67	01	10	13	6B	2.000	PTDEC
P174	B	66	01	10	13	6B	2.000	PTDEC
P458	B	59	01	10	13	6B	1.000	PTDEC
P086	P	59	01	03	13	6B	2.000	PTDEC
P228	B	59	01	10	13	6B	1.000	PTDEC
P122	B	62	01	10	13	6B	0.060	PTDEC
P216	N	78	01	10	13	6B	2.000	PTDEC
P244	P	78	01	06	13	6B	2.000	PTDEC
P397	B	79	15	02	39	14	0.250	PT
P411	P	74	01	06	13	6B	2.000	PTDEC
P543	P	75	01	06	13	6B	2.000	PTDEC
P409	P	76	01	06	13	6B	2.000	PTDEC
P304	B	77	01	03	13	6B	2.000	PTDEC
P165	P	23	01	01	01	9V	2.000	PT
P353	N	23	01	01	01	9V	2.000	PT
P117	K	23	01	01	01	9V	1.000	PT
P384	K	23	01	01	01	9V	2.000	PT

[a]B, Children's Hospital Bangkok; C, Cholburi Hospital; K, Khonkaen Hospital; N, Maharaj Nakorn Ratchasima Hospital; P, Phra Buddha Chinaraj Hospital; S, Hatyai Hospital.
[b]Resistance profile: P, penicillin; T, trimethoprim-sulfamethoxazole; D, doxycycline; E, erythromycine; C, chloramphenicol.
[c]NT, nontypeable
[d]MICs are in micrograms per milliliter

RFEL analysis of 53 penicillin-resistant pneumococcal isolates that were collected from children with acute respiratory tract infections in five distinct regions divided the strains into 27 distinct types. These RFEL types represented 13 clusters, i.e., types shared by two or more strains, and 14 unique types. Thirty-nine strains shared RFEL types with at least one other strain (74%). The degree of clustering among the penicillin-resistant isolates was comparable with that of data obtained in The Netherlands (17), a country with a very low prevalence of penicillin resistance (<1%). These data clearly demonstrate that the transmission behavior of these strains is comparable in both countries. The cluster size varied from two (nine clusters) to 11 strains (one cluster). The most predominant RFEL cluster, representing 21% of the isolates, was identical to the Spanish pandemic clone 23F (RFEL type 15) (26). In addition, the second largest RFEL cluster (RFEL type 23; four isolates) matched the Spanish-French international clone 9V (13). These data indicate that the pandemic clones 23F and 9V, which are widely spread throughout the world (15, 16, 38), are the most predominant multidrug-resistant pneumococcal clones in Thailand.

Although the five regions were not equally represented by pneumococcal isolates, geographical distribution of the genotypes among the five Thai regions demonstrated that only the RFEL clusters 66 (n = 2), 67 (n = 2), 68 (n = 2), and 70 (n = 2) were observed in single areas of the country, whereas the other nine clusters represented isolates collected in two or more districts. These data clearly suggest the national spread of the majority of the RFEL clusters. The majority of the RFEL types (24 of 27 types) were Thailand specific, as they did not match with any of the 107 RFEL types representing 15 other countries. In addition, the PBP genotypes also suggest a Thailand-specific origin: the majority of the PBP types (12 of 16 types) were not observed in the international collection. Nevertheless, since the pandemic clones 23F and 9V are most predominantly present in Thailand, we conclude that these imported clones are primarily responsible for the high prevalence of pneumococcal penicillin resistance in this country.

The increasing rate of antibiotic resistance in *S. pneumoniae* complicates the elimination of pneumococci by therapy and strongly supports the application of new vaccine strategies. Conjugate capsular vaccines contain a limited number of capsular serotypes, linked to a

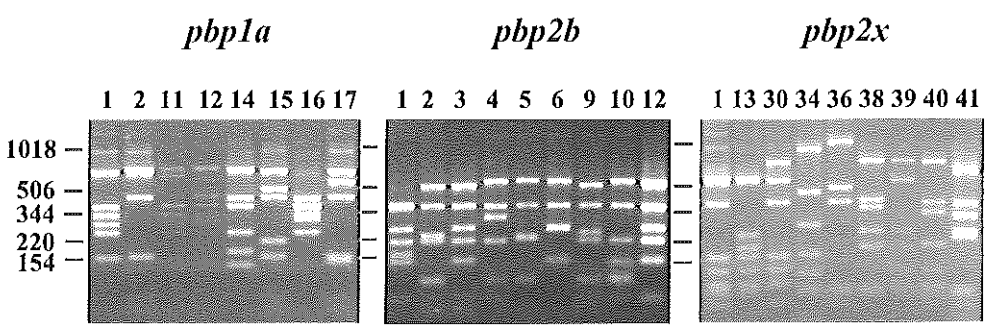

Fig. 3. DNA fingerprint patterns of the *pbp1a* (n = 8), *pbp2b* (n = 9), and *pbp2x* (n = 9) genotypes represented by the 53 Thai penicillin-resistant pneumococcal strains. Lane numbers indicate PBP genotype codes. Numbers at the left indicate the sizes of standard DNA fragments in base pairs.

carrier protein (3, 9). Although the results of early trials with these vaccines look promising, care should be taken since several investigators have observed horizontal transfer of capsular genes (2, 15–17). Horizontal transfer of capsular genes may interfere with vaccination programs in the long run if (antibiotic-resistant) strains with a vaccine-type capsule switch to nonvaccine capsular types. In Thailand, horizontal transfer of capsular genes appears to occur frequently. Six of the 13 RFEL clusters consisted of two or more distinct serotypes. The pandemic clone 23F (RFEL cluster 15; 11 strains), harbored three distinct serotypes, 23F, 19F, and 14. Based on the serotype distribution of the 53 penicillin-resistant pneumococci, 89% of the strains display capsular types that are covered by the 7-valent conjugate vaccine. However, it is important to notice that in the collection investigated in this study the number of high-level penicillin-resistant isolates is over represented. Since the number of non-vaccine serotypes is higher in the group of intermediate-level resistant isolates, vaccine coverage of penicillin-resistant pneumococci (MIC of ≥0.1 μg/ml) is therefore expected to be lower than calculated.

The emergence of resistant strains and the rapid spread of resistant clones raise the need for an effective global surveillance system. Detailed studies on the epidemiology and epidemic behavior of (multi)resistant pneumococci will assist in identifying emerging clones. To this respect, close collaboration between the laboratories sharing interests in pneumococcal molecular epidemiology is of utmost importance. Extensive collaboration can be facilitated by the establishment of a freely accessible electronic network. Such a network can be used to exchange epidemiological information. Consequently, this network can be used to construct and distribute an international data library containing DNA fingerprints of (multi)resistant

pneumococcal strains. This approach will facilitate adequate worldwide monitoring of the epidemiology of emerging (multi)resistant pneumococcal strains.

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Chapter 5

Molecular characterization of penicillin-binding protein genes of penicillin-nonsusceptible *Streptococcus pneumoniae* isolated in The Netherlands

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SUMMARY

Recently, a nation-wide molecular epidemiologic survey of penicillin-nonsusceptible *Streptococcus pneumoniae* has been performed in The Netherlands. In the current study, we analyzed the *pbp* genes from these clinical isolates in detail. The pneumococcal strains were selected on the basis of differences in restriction fragment length polymorphism (RFLP) patterns of the complete genes *pbp1a, pbp2b* and *pbp2x*, representing 8, 7 and 10 distinct patterns, respectively. We characterized specific gene parts of *pbp1a* (nucleotide position 1741 to 2229), *pbp2b* (nucleotide position 1211 to 1714) and *pbp2x* (nucleotide position 1241 to 1786). Classification based upon sequence analysis of these *pbp* fragments correlated well with the classification according to RFLP analysis. Sequence analysis of *pbp2b* enables a refinement of the classification based on RFLP analysis. However, sequence analysis of *pbp1a* and *pbp2x* was less discriminatory compared to RFLP analysis. The mutations in the *pbp* sequences of the Dutch isolates invariably matched with the mutations described in *pbp* sequences of penicillin-nonsusceptible pneumococci isolated in other countries. This observation supports the hypothesis that multiple clones of penicillin-resistant pneumococci have been imported and spread in The Netherlands. Interestingly, novel combinations of mosaic structures were also identified indicating horizontal exchange of *pbp* gene parts among penicillin-nonsusceptible pneumococci.

INTRODUCTION

Streptococcus pneumoniae is a common cause of serious and life-threatening infections, such as pneumonia, bacteremia and of noninvasive infections such as otitis media and sinusitis. Ever since the beginning of the antibiotic era, penicillin has been the first choice drug against pneumococcal infections. However, in the last 20 years, increasing numbers of penicillin-nonsusceptible pneumococcal clinical isolates with rising MIC values have been isolated (11).

Penicillin resistance in *S. pneumoniae* has emerged through the development of altered penicillin-binding proteins (PBPs), which show a decreased affinity for penicillin and other β-lactam antibiotics. PBPs are membrane-bound D,D-peptidases, which catalyze the transpeptidation reaction that cross-links the peptidoglycan of the bacterial cell wall. The β-lactam antibiotics interact with the PBPs and form a covalent penicilloyl complex via the

active-site serine resulting in an inactivated enzyme. In penicillin-susceptible pneumococci, this interaction results in cellular lysis (4, 11). *S. pneumoniae* contains six PBPs: the high molecular weight proteins PBP1a, PBP1b, PBP2a, PBP2b and PBP2x, and the low molecular weight protein PBP3. In clinical isolates, altered PBP2b and PBP2x are the primary resistance determinants, i.e. they confer low-level resistance when introduced into sensitive strains, whereas alterations in PBP1a contribute to an increase in resistance levels (10, 13). The alterations in the PBPs are due to the formation of a mosaic structure of the genes, presumably as a result of horizontal gene transfer. The *pbp* genes of nonsusceptible isolates are composed of mosaic blocks that share identity with *pbp* genes from sensitive isolates and blocks that are identical to *pbp* genes from *Streptococcus mitis, Streptococcus sanguis* and *Streptococcus oralis* (23). It is therefore suggested that the mosaic *pbp* genes are the result of interspecies recombination. Some mosaic *pbp* genes are composed of a single block from viridans streptococci, whereas others are the result of complex multiple recombination events involving several donors (6). The presence of an enormous diversity of mosaic *pbp* genes suggests that resistance to penicillin has arisen on many independent occasions. These recombination events have resulted in multiple amino acid substitutions in the PBPs. Several amino acids are essential for the interaction of the PBPs with β-lactam antibiotics. Substitutions of these amino acids often result in a reduction of affinity and consequently in reduced penicillin susceptibility. Most mutations which confer resistance occur close to the motifs SXN or KT/SG, or close to the active-site serine which is part of the conserved STMK motif (11, 12).

We have recently investigated the epidemiologic characteristics of penicillin-nonsusceptible pneumococci in The Netherlands in a nation-wide survey (16). The penicillin-nonsusceptible clinical isolates have been analyzed by restriction fragment length polymorphism (RFLP) analysis of the genes *pbp1a*, *pbp2b* and *pbp2x*. In this study, we sequenced gene parts of *pbp1a*, *pbp2b* and *pbp2x* representing distinct RFLP patterns. These data were used to investigate the correlation between *pbp* RFLP analysis and *pbp* sequence analysis, and to identify the molecular nature of pneumococcal penicillin resistance in The Netherlands.

MATERIALS AND METHODS

Bacterial strains, growth conditions and growth medium. Penicillin-nonsusceptible pneumococcal strains were isolated from patients in The Netherlands between March 1995

and March 1996. Their epidemiological characteristics have been described previously (16). The majority of the strains were isolates from patients with pneumococcal pneumonia. Clinical isolates representing distinct restriction fragment length polymorphism patterns of *pbp1a, pbp2b* and *pbp2x*, respectively, were used for nucleotide sequence analysis of their *pbp* genes. The pneumococcal strains reported in this study are listed in Fig. 1 to 3. The penicillin MICs of these strains ranged from 0.12 to 2 μg/ml. Bacteria were cultured at 37°C on Columbia agar supplemented with 5% sheep blood (Oxoid, Basingstoke, United Kingdom) in an atmosphere of increased CO_2. Bacterial colonies were inoculated in Todd-Hewitt broth (Difco laboratories, Detroit, USA) supplemented with 0.5% yeast extract (Difco laboratories; THY broth) and grown at 37°C.

PBP genotyping. Genetic polymorphisms of the penicillin resistance genes *pbp1a, pbp2b,* and *pbp2x* were investigated by restriction fragment length polymorphism (RFLP) analysis as described previously (16). Briefly, chromosomal DNA was extracted with cetyl trimethylammonium bromide (26). PCR amplification of the PBP-encoding genes was performed in 50 μl reactions containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (wt/vol) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 10 pmol of the individual primers, 0.5 U of DNA polymerase (Eurogentec, Liège, Belgium), and 10 ng of purified chromosomal DNA. Cycling was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, USA) and consisted of the following steps: predenaturation at 94°C for 1 min, 30 cycles of 1 min at 94°C, 1 min at 52°C, 2 min at 72°C, and a final extension at 72°C for 3 min. The primers used to amplify the genes *pbp1a, pbp2b,* and *pbp2x* were described previously (see Table 1). The amplification products (5 μl) were digested with the restriction endonuclease *Hinf*I and separated by electrophoresis in 2.5% agarose gels. Gels were scanned and printed with the Geldoc 2000 system (Biorad, Veenendaal, The Netherlands). The different PBP genotypes are represented by a three-number code (e.g., 06-14-43), referring to the RFLP patterns of the genes *pbp1a* (pattern 6), *pbp2b* (pattern 14), and *pbp2x* (pattern 43), respectively. The PBP RFLP codes used in this study refer to those described previously (16).

Nucleotide sequence analysis. Nucleotide sequencing was performed on parts of the penicillin-binding domain of *pbp1a*, of *pbp2b*, and of *pbp2x*, respectively, from the pneumococcal strains mentioned in Fig. 1 to 3. The primer pairs Pbp1a.fw/Pbp1a.rev,

Pbp2b.fw/Pbp2b.rev and Pbp2x.fw/Pbp2x.rev were used to amplify nucleotides 1723 to 2350 of *pbp1a*, nucleotides 1199 to 1734 of *pbp2b*, and nucleotides 1217 to 1796 of *pbp2x*, respectively (Table 1). The numbering is based on published data for strain R6 [*pbp1a* (20), *pbp2b* (9), *pbp2x* (18)]. PCR-amplification was performed as described above in 100 µl reactions with 50 ng of purified pneumococcal chromosomal DNA. The PCR products were separated by electrophoresis in 1% agarose gels, cut from gel and purified with the QIAquick Gel Extraction kit (Qiagen, Westburg, Leusden, The Netherlands). Purified templates were sequenced with the Prism Ready Reaction Sequence Kit (Perkin Elmer, Roosendaal, The Netherlands) and 50 pmol of the primers M13.fw and M13.rev (Table 1). Sequencing was performed on an Applied Biosystems Prism 377 (PE Applied Biosystems, Nieuwerkerk, The Netherlands). The nucleotide and deduced amino acid sequences were compared to the published sequence of *pbp1a* (20), *pbp2b* (9) and *pbp2x* (18) from the penicillin-susceptible strain R6 with BioEdit Sequence Alignment Editor (15). With the BLAST algorithm (1), the nucleotide sequences were analyzed for similarity to sequences deposited in the April 2000 version of the nucleotide database at the National Center for Biotechnology Information (Washinton D.C., USA).

Table 1. Primers used in this study. The underlined sequence parts of the primers Pbp1a.fw, Pbp1a.rev, Pbp2b.fw, Pbp2b.rev, Pbp2x.fw, Pbp2x.rev represent M13-based sequences.

primer name	primer sequence	reference
Pn1Aup	CGGCATTCGATTTGATTCGCTTCT	(5)
Pn1Adown	CTGAGAAGATGTCTTCTCAGG	(5)
Pn2Bup	GATCCTCTAAATGATTCTCAGGTGG	(8)
Pn2Bdown	CAATTAGCTTAGCAATAGGTGTTGG	(8)
Pn2Xup	CGTGGGACTATTTATGACCGAAATGG	(22)
Pn2Xdown	AATTCCAGCACTGATGGAAATAAACATATTA	(22)
Pbp1a.fw	TGTAAAACGACGGCCAGTCAAAGTCTCAAATCAGCAAG	this study
Pbp1a.rev	CAGGAAACAGCTATGACCGTTGTGTTACTTGAAATGGC	this study
Pbp2b.fw	TGTAAAACGACGGCCAGTCTGAAAAGTTATTTCAATTC	this study
Pbp2b.rev	CAGGAAACAGCTATGACCAAWCCAGTWGAYTCATCTGG	this study
Pbp2x.fw	TGTAAAACGACGGCCAGTAAAATGGGAGATGCTAC	this study
Pbp2x.rev	CAGGAAACAGCTATGACCTGGATACCTGAATAATG	this study
M13.fw	TGTAAAACGACGGCCAGT	
M13.rev	CAGGAAACAGCTATGACC	

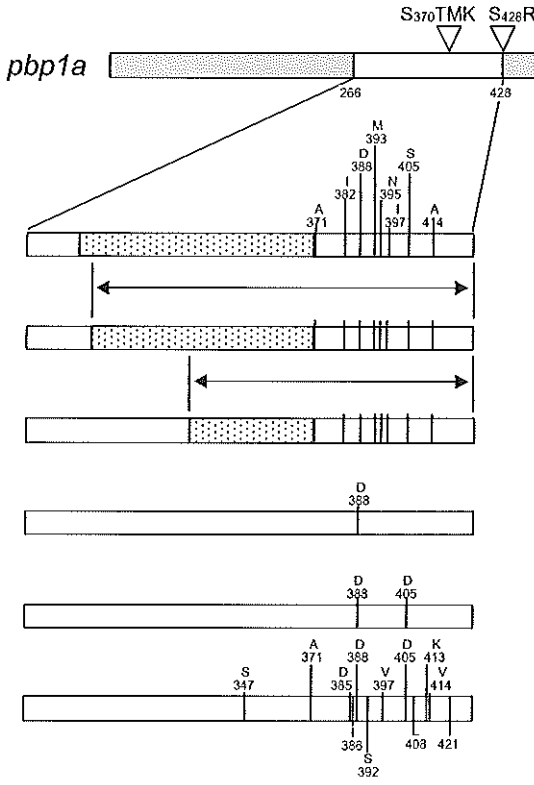
Nucleotide sequence accession numbers. The *pbp1a* sequence data for the following clinical isolates were submitted to the EMBL/GenBank databases under the indicated accession numbers (acc. nos.): isolate 950225, AJ403974; isolate 960036, AJ403975; isolate

950710, AJ403976; isolate 950421, AJ403977; isolate 950423, AJ403978; isolate 950473, AJ403979; isolate 960027, AJ403980 and isolate 950035, AJ403981. The *pbp2b* sequence data for the following clinical isolates have been submitted to the EMBL/GenBank databases under the indicated acc. nos.: isolate 950279, AJ278222; isolate 950961, AJ278223; isolate 950901, AJ278224; isolate 950138, AJ278225; isolate 960118, AJ278226; isolate 960035, AJ278227; isolate 960036, AJ278228; isolate 960030, AJ278229; isolate 954073, AJ278230 and isolate 960097, AJ278231. The *pbp2x* sequence data for the following clinical isolates have been submitted to the EMBL/GenBank databases under the indicated acc. nos.: isolate 950181, AJ278232; isolate 950138, AJ278233; isolate 950423, AJ278234; isolate 960036, AJ278235; isolate 950454, AJ278236; isolate 950961, AJ278237; isolate 950637, AJ278238; isolate 950925, AJ278239, isolate 960097, AJ278240; and isolate 960165, AJ278241.

RESULTS

Sequence heterogeneity in *pbp1a* of penicillin-nonsusceptible pneumococcal isolates. We sequenced nucleotides 1741 to 2229 of *pbp1a* from 44 Dutch clinical pneumococcal isolates representing eight distinct *pbp1a* RFLP types. The *pbp1a* nucleotide sequences from strains representing identical *pbp1a* RFLP types were invariably identical (data not shown). A representative strain of each *pbp1a* RFLP type was chosen for sequence comparison. Fig. 1 provides a schematic illustration of the mosaic structures of *pbp1a*.

The nucleotide sequences and deduced amino acid sequences of *pbp1a* obtained from the RFLP type 1 and 10 strains were identical (Fig. 1). Nucleotides 1741 to 1789 differed from the penicillin-susceptible strain R6 by one synonymous substitution, nucleotides 1790 to 2039 were identical to *pbp1a* from *S. mitis* strain B6 (acc. no. AJ002290) (14), and nucleotides 2040 to 2229 differed from strain R6 by 11 synonymous substitutions and 11 nonsynonymous substitutions resulting in eight amino acid changes (Thr371Ala, Leu382Ile, Glu388Asp, Ile393Met, His395Asn, Glu397Ile, Asn405Ser, Gly414Ala) (Fig. 1). The *pbp1a* sequences of RFLP types 1 and 10 matched with the *pbp1a* sequence of *S. pneumoniae* strain D isolated in Spain (21) and *S. pneumoniae* strains #5/H31 and #17/246 isolated in Japan (3). Part of *pbp1a* of the RFLP type 3 strain (nucleotides 1880 to 2229) and part of *pbp1a* of the RFLP type 6 strain [nucleotides 1920 to 2229 except for five synonymous substitutions and one nonsynonymous substitution (Ala371Thr)] were identical to *pbp1a* of RFLP type 1 and 10 strains (Fig. 1). The *pbp1a* regions of the RFLP type 2 and 5 strains were identical and



strain	penicillin MIC (µg/ml)	type
950225	2	1
960035	2	10
950710	2	3
950473	0.25	6
960036	0.12	2
950423	0.25	5
950421	0.25	4
960027	2	7

Fig. 1. Schematic representation of *pbp1a*. The STMK-motif, SRN-motif and KTG-motif are depicted. The sequenced part is indicated by the open block. The mosaic structures in the deduced amino acid sequences of strains representing distinct RFLP types are shown below. Open blocks indicate DNA from susceptible pneumococcal strains and dotted blocks are homologous to *S. mitis* strain B6 DNA (14). Vertical lines indicate amino acid substitutions. Arrows indicate the identity between DNA sequences derived from genes representing distinct types. The pneumococcal strains, penicillin MICs, and *pbp1a* types are also listed.

differed from strain R6 by two synonymous substitutions and a single nonsynonymous substitution (Glu388Asp) (Fig. 1). Interestingly, this substitution occurred in the deduced amino acid sequences from all isolates analyzed in this study. The *pbp1a* sequences of RFLP type 2 and 5 strains were identical to *S. pneumoniae* strain C, a penicillin-susceptible strain isolated in South Africa (21). The *pbp1a* sequence of the RFLP type 4 strain differed in seven additional synonymous substitutions and one additional nonsynonymous substitution (Asn405Asp) (Fig. 1). *pbp1a* of RFLP type 2, 4, and 5 strains were highly homologous to *pbp1a* of *S. pneumoniae* strain #23/HSB21 isolated in Japan (3). The *pbp1a* nucleotide and deduced amino acid sequence of the RFLP type 7 strain differed significantly from the previous sequences, and differed from strain R6 by 52 synonymous substitutions and 13 nonsynonymous substitutions resulting in 12 amino acid changes (Ala347Ser, Thr371Ala,

Gly385Asp, Val386Ile, Glu388Asp, Thr392Ser, Glu397Val, Asn405Asp, Val408Leu, Arg413Lys, Gly414Val, Leu421Ile) (Fig. 1). The partial *pbp1a* sequence of the RFLP type 7 was homologous to *S. pneumoniae* strain #14/Z42 isolated in Japan (3). Interestingly, the amino acid substitution Thr371Ala was found in all high-level penicillin-resistant strains.

Sequence heterogeneity in *pbp2b* of penicillin-nonsusceptible isolates. We sequenced nucleotides 1211 to 1714 of *pbp2b* from 57 Dutch clinical pneumococcal isolates representing seven distinct *pbp2b* RFLP types. The *pbp2b* sequences from strains representing identical *pbp2b* RFLP types were identical for RFLP types 2, 4 and 5, respectively (data not shown). In contrast, the *pbp2b* nucleotide sequences from strains representing RFLP type 1 could be divided into two sequence types designated type 1A and 1B. In addition, the *pbp2b* nucleotide sequences from strains representing RFLP type 3 could be divided into three sequence types designated type 3A, 3B and 3C. RFLP types 7 and 8 were represented by one strain each. A representative strain of each RFLP type and sequence-type, respectively, was chosen for sequence comparison (Fig. 2).

The *pbp2b* sequence of the RFLP type 1A strain was identical to *pbp2b* from the penicillin-resistant *S. sanguis* strain 1907 (acc. no. M32226), except for one synonymous substitution (Fig. 2). In addition, this *pbp2b* fragment was identical to *pbp2b* of *S. pneumoniae* strains SP1470 isolated in France (acc. no. AF210766), M15 isolated in the United Kingdom (acc. no. AJ243054), 577 isolated in the United Kingdom (acc. no. AJ243053) and 56762 isolated in South Africa (acc. no. U20080). *pbp2b* of the RFLP type 1B strain was in part identical to the *pbp2b* sequence of the RFLP type 3A strain (nucleotides 1211 to 1402) and in part to the *pbp2b* sequence of the RFLP type 1A strain (nucleotides 1403 to 1714) (Fig. 2). The *pbp2b* fragment of the RFLP type 3A strain was identical to *pbp2b* of *S. mitis* strain B6 (acc. no. AJ002289), except for one nonsynonymous mutation (Ile382Val) (Fig. 2). In addition, this *pbp2b* fragment was identical to penicillin-resistant *S. oralis* strain 5296 (acc. no. M32228), except for one synonymous mutation and one nonsynonymous mutation (Ile382Val). The *S. pneumoniae* strain 52328 isolated in South Africa (acc. no. U20073) also contained the same *pbp2b* sequence, except for two synonymous mutations. The *pbp2b* fragment of the pneumococcal RFLP type 3B strain was identical to that of the RFLP type 3A strain, with the exception of the fragment formed by nucleotides 1227 to 1279, which was identical to that in the penicillin-susceptible strain R6 (Fig. 2). *pbp2b* of the RFLP type 3C strain was in part homologous to the R6 sequence (nucleotides 1211 to 1391) differing in 6 synonymous

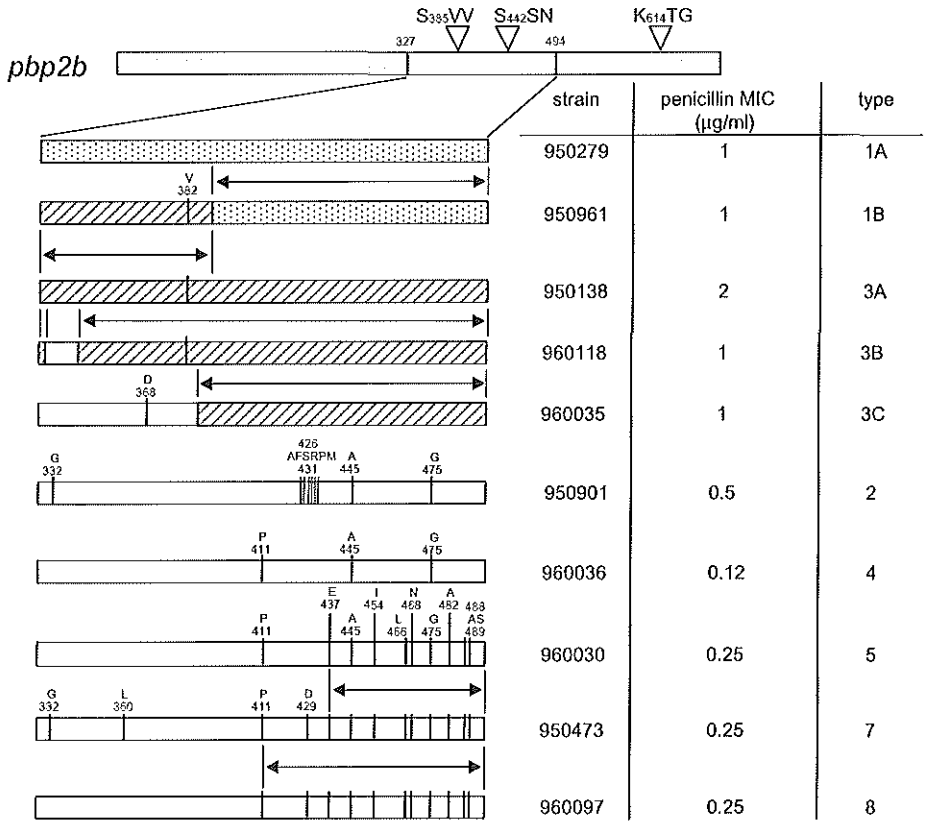


Fig. 2. Schematic representation of *pbp2b*. The SVV-motif, SSN-motif and KTG-motif are depicted. The sequenced part is indicated by the open block. The mosaic structures in the deduced amino acid sequence of strains representing distinct RFLP types are shown below. Open blocks indicate DNA from susceptible pneumococcal strains, dotted blocks are homologous to *S. mitis* strain B6 (acc. no. AJ 002289) and *S. sanguis* strain 1907 DNA (acc. no. M32226), and striped blocks are homologous to penicillin-resistant *S. oralis* strain 5296 DNA (acc. no. M32228). Vertical lines indicate amino acid substitutions. Arrows indicate the identity between DNA sequences derived from genes representing distinct types. The pneumococcal strains, penicillin MICs, and *pbp2b* type are also listed.

mutations and one nonsynonymous mutation (Glu368Asp), and in part identical to *pbp2b* of RFLP type 3A and 3B strains, except for one synonymous mutation (Fig. 2). *pbp2b* of the RFLP type 2 strain contained 26 synonymous substitutions and 9 nonsynonymous (Glu332Gly, Gln426Ala, Ala427Phe, Tyr428Ser, Gly429Arg, Ser430Pro, Phe431Met, Thr445Ala, Glu475Gly) substitutions compared to the R6 sequence. The *pbp2b* fragment of this strain was identical to *S. pneumoniae* strains DN87/669 isolated in the United Kingdom (acc. no. M25521) and 8249 isolated in South Africa (acc. no. M25520), except for one synonymous substitution (7). The *pbp2b* fragment of the RFLP type 4 strain contained 22 synonymous substitutions and three nonsynonymous (Ser411Pro, Thr445Ala, Glu475Gly)

substitutions compared to the R6 sequence and was not identical to any of the published sequences present in databases (Fig. 2). The *pbp2b* fragment of the RFLP type 5 strain differed in 49 synonymous mutations and 11 nonsynonymous mutations resulting in 10 amino acid changes (Ser411Pro, Gln437Glu, Thr445Ala, Leu454Ile, Gly466Leu, Ser468Asn, Glu475Gly, Gly482Ala, Thr488Ala, Ala489Ser) from *pbp2b* of strain R6 and was identical to *pbp2b* of *S. pneumoniae* strain SP 1513 isolated in France (acc. no. AF210762) (Fig. 2). Part of the *pbp2b* fragment of the RFLP type 5 strain (nucleotides 1460 to 1714) was homologous to *pbp2b* of *S. mitis* strain NCTC 11189 (acc. no. Z22183). In this fragment, we observed 11 synonymous mutations and 4 amino acid substitutions (Thr445Ala, Gly466Leu, Ser468Asn, Ala480Ser) compared to the *S. mitis* sequence, while 40 synonymous mutations and the 11 nonsynonymous mutations (Ser411Pro, Gln437Glu, Thr445Ala, Leu454Ile, Gly466Leu, Ser468Asn, Glu475Gly, Gly482Ala, Thr488Ala, Ala489Ser) were observed compared to the R6 sequence (Fig. 2). *pbp2b* of the RFLP type 7 and 8 strains were in part identical to *pbp2b* from the RFLP type 5 strain (Fig. 2). The *pbp2b* sequence of the RFLP type 7 strain differed in nucleotides 1211 to 1455 from the R6 sequence by eight synonymous substitutions and two nonsynonymous substitutions (Glu332Gly, Ile360Leu), was identical to *pbp2b* of the RFLP type 8 strain for nucleotides 1456 to 1714, and, except for two synonymous mutations, was identical to *pbp2b* of the RFLP type 5 strain for nucleotides 1528 to 1714 (Fig. 2). *pbp2b* of the RFLP type 8 strain was identical to *pbp2b* of R6 for nucleotides 1211 to 1455 and to *pbp2b* of the RFLP type 7 strain for nucleotides 1456 to 1714 (Fig. 2). The Thr445Ala substitution was found in all penicillin-nonsusceptible strains we investigated. In addition, the Glu475Gly substitution was found in all *pbp2b* sequence types of our strain collection. Six consecutive substitutions at residues 426 to 431 were found in *pbp2b* RFLP type 2 only.

Sequence heterogeneity in *pbp2x* of penicillin-nonsusceptible strains. We sequenced nucleotides 1241 to 1786 of *pbp2x* from 53 Dutch clinical pneumococcal isolates representing 10 distinct RFLP types. The *pbp2x* nucleotide sequences from strains representing identical *pbp2x* RFLP types were identical as far as RFLP type 1, 4, 6, 8, and 20 was concerned, except for two or less synonymous point mutations observed in RFLP type 1. The RFLP types 7, 13, 22, 25 and 31 were represented by single strains. A representative strain for each *pbp2x* RFLP type was chosen for sequence comparison (Fig. 3).

The *pbp2x* DNA fragments of the RFLP type 1, 8 and 13 strains were identical in nucleotide and deduced amino acid sequence (Fig. 3). This sequence type shared identity with *pbp2x* of

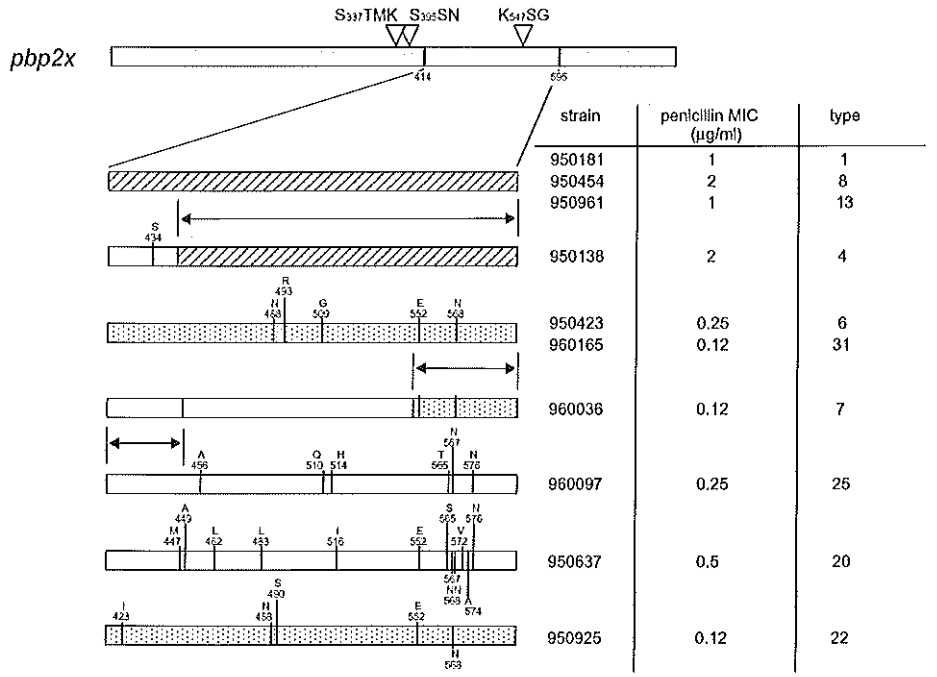


Fig. 3. Schematic representation of *pbp2x*. The STMK-motif, SSN-motif and KSG-motif are depicted. The sequenced part is indicated by open block. The mosaic structures in the deduced amino acid sequences of strains representing distinct RFLP types are shown below. Open blocks indicate DNA from susceptible pneumococcal strains, dotted blocks are homologous to *S. mitis* strain 10712 DNA (acc. no. X78216), and striped blocks are homologous to *S. oralis* strain U5 DNA (acc. no. Y10536), *S. mitis* strains 476 (acc no. Y10534) and 197 (acc. no. Y10533) DNA. Vertical lines indicate amino acid substitutions. Arrows indicate the identity between the DNA sequences derived from genes representing distinct types. The pneumococcal strains, penicillin MICs, and *pbp2x* type are also listed.

S. oralis strain U5 (acc. no. Y10536), and *pbp2x* of *S. mitis* strains 476 (acc. no. Y10534) and 197 (acc. no. Y10533). In addition, the *pbp2x* sequence of RFLP type 1, 8 and 13 strains was identical to *pbp2x* of *S. pneumoniae* strain 577 isolated in the United Kingdom (19) and *S. pneumoniae* strains 34/H31 and 31/KU5 isolated in Japan (2). The *pbp2x* fragment from the RFLP type 4 strain was identical for the nucleotides 1319 to 1786 with *pbp2x* from RFLP type 1, 8 and 13 strains. The 5' part of the sequence (nucleotides 1211 to 1318) differed from *pbp2x* of strain R6 by 14 synonymous mutations and one nonsynonymous mutation (Ala434Ser) (Fig. 3). The *pbp2x* fragment of the RFLP type 6 strain was highly homologous to *pbp2x* of *S. mitis* strain 10712 (acc. no. X78216), and differed in 10 synonymous substitutions and 5 nonsynonymous substitutions (Asp488Asn, Lys493Arg, Ser509Gly, Gln552Glu, Tyr568Asn) (Fig. 3). The *pbp2x* fragment of the RFLP type 6 strain was identical to *pbp2x* of *S. pneumoniae* strain F1 isolated in France (acc. no. AJ238581), except

for two synonymous substitutions. *pbp2x* of the RFLP type 31 strain was identical to *pbp2x* RFLP type 6 sequences, except for five synonymous substitutions (Fig. 3). The *pbp2x* sequence of the RFLP type 25 strain differed from *pbp2x* of the R6 sequence in 56 synonymous substitutions and 6 nonsynonymous substitutions (Val456Ala, Leu510Gln, Asn514His, Leu565Thr, Asp567Asn, Ser576Asn) (Fig. 3). The *pbp2x* sequence of the RFLP type 25 strain was identical to *pbp2x* of *S. pneumoniae* strain 53139/72 isolated in Papua New Guinea (19). *pbp2x* of the RFLP type 7 strain was in part homologous to *pbp2x* of the RFLP type 25 strain, in part to *pbp2x* of R6, and in part to *pbp2x* of the RFLP type 6 and 31 strains (Fig. 3): nucleotides 1241 to 1345 of *pbp2x* of the RFLP type 7 strain were identical to *pbp2x* of the RFLP type 25 strain except for two synonymous mutations, nucleotides 1346 to 1650 were identical to *pbp2x* of strain R6 except for two synonymous mutations, and nucleotides 1651 to 1786 were identical to *pbp2x* of strains representing RFLP types 6 except for two synonymous mutations. The *pbp2x* sequence of the RFLP type 20 strain differed from R6 in 60 synonymous substitutions and 12 nonsynonymous substitutions (Gln447Met, Ser449Ala, Ile462Leu, Ile483Leu, Val516Ile, Gln552Glu, Leu565Ser, Asp567Asn, Tyr568Asn, Ala572Val, Ser574Ala, Ser576Asn) (Fig. 3). The *pbp2x* RFLP type 20 sequence was identical to *pbp2x* of *S. pneumoniae* strains SP1258 isolated in France (acc. no. AF210756) and 669 isolated in the United Kingdom (acc. no. X65133) (19). The sequenced *pbp2x* fragment of the RFLP type 22 strain was homologous to *pbp2x* of *S. mitis* strain 10712 (acc. no. X78216) although 32 synonymous and 5 nonsynonymous substitutions (Val423Ile, Asp488Asn, Thr490Ser, Gln552Glu, Tyr568Asn) were present. *pbp2x* of the RFLP type 22 strain was identical to *pbp2x* of *S. pneumoniae* strain SP1513 isolated in France (acc. no. AF210754), except for one nonsynonymous substitution (Asn567His).

DISCUSSION

Pneumococcal isolates express six PBPs. Many penicillin-nonsusceptible isolates are modified in PBP1a, PBP2b and PBP2x only (12). If one or more amino acids change in the conserved amino acid motifs SXXK (with active site serine), SXN and K(H)T(S)G or adjacent amino acids, penicillin and other β-lactam antibiotics are often unable to bind efficiently to PBPs, which results in resistance.

RFLP analysis has previously been performed to characterize *pbp1a*, *pbp2b* and *pbp2x* from penicillin-nonsusceptible strains isolated in The Netherlands that displayed a wide range of

MIC levels (0.12 to 2 µg/ml) (16). In this study, we characterized parts of the DNA sequences of *pbp1a*, *pbp2b* and *pbp2x* of these clinical isolates. The classification based on sequence analysis of the *pbp* fragments correlated well with the classification according to *pbp* RFLP analysis. Sequence analysis of the *pbp2b* fragment (nucleotides 1211 to 1714) enables a refinement of the classification based on RFLP analysis of the complete *pbp2b* gene. The *pbp2b* RFLP types 1 and 3 were divided into sequence types 1A and 1B, and 3A, 3B and 3C, respectively. However, the classification of the *pbp2b* RFLP types 2, 4, 5, 7 and 8 was maintained after sequence analysis. We were unable to differentiate within the *pbp1a* RFLP types by *pbp1a* sequence analysis of nucleotides 1741 to 2229. Moreover, strains with *pbp1a* RFLP types 1 and 10, and strains with RFLP types 2 and 5 were classified to the same sequence type, respectively. In addition, we were not able to subdivide *pbp2x* RFLP types by sequence analysis of *pbp2x* nucleotides 1241 to 1786 and strains with *pbp2x* RFLP type 1, 8 and 13 were classified to the same sequence type.

Most of the *pbp* sequences characterized in this study matched with *pbp* sequences from strains isolated in other countries. Martin et al. described *pbp1a* sequence parts of eight resistant isolates (21) and Asahi et al. classified *pbp1a* and *pbp2x* sequences from clinical isolates into five sequence types (I to V) each (2, 3). All *pbp1a* sequence types identified in this study could be matched with *pbp1a* sequences described by both research groups. The *pbp2x* sequences of RFLP type 1, 8 and 13 strains were identical to *pbp2x* sequence types IV and V (2). In addition, the *pbp2x* sequences of the RFLP type 20 and 25 strains matched with sequences described by Laible et al. (19). Dowson and colleagues have divided *pbp2b* from penicillin-resistant isolates in two classes, A and B (7). *pbp2b* RFLP type 1A was similar to class B *pbp2b*, and *pbp2b* RFLP type 2 belongs to class A *pbp2b*. All other *pbp2b* RFLP types described in this study did not match any of the classes A or B, pointing to the limitations of Dowson's classification.

Interestingly, penicillin-nonsusceptible strains with *pbp* sequences that were highly homologous to *pbp1a* sequences from susceptible strains were intermediately resistant. In contrast, penicillin-nonsusceptible strains with a high degree of heterogeneity in their *pbp* sequences compared to susceptible strains have often MIC levels of ≥1 µg/ml. The difference in penicillin susceptibility between strains belonging to the same sequence type may be due to amino acid alterations in non-sequenced parts of the penicillin-binding domain and in other *pbp* genes.

All prominent PBP1a amino acid substitutions in penicillin-nonsusceptible isolates described by Smith *et al.* (24) were also observed in this study. The amino acid change Asn405Asp was previously identified as being one of the prominent amino acid substitutions in *pbp1a* of resistant isolates (24). In addition, we confirmed that the Thr371Ala substitution in PBP1a was important for high-level resistance (3), since all high-level penicillin-resistant strains contained this substitution. The PBP1a amino acid substitution Glu388Asp, which has been identified in all penicillin-nonsusceptible isolates in this study, also occurs in susceptible strains. This substitution is probably not able to confer penicillin resistance (24). The amino acid substitutions found in PBP2b in strains of our collection confirmed the previously described amino acid substitutions in penicillin-resistant isolates (8, 25, 27). The Thr445Ala substitution was found in all penicillin-nonsusceptible strains adjacent to the Ser443-Ser-Asn motif, and its importance has previously been noted by Dowson and coworkers (8). The Asn residue of this motif has been proposed to form a hydrogen bond with the carbonyl group of the R1 side chain of penicillin, and the Thr445Ala substitution presumably disrupts this hydrogen bond (8). The significance of Glu475Gly has been noted by Smith and Klugman (25), and was found in all *pbp2b* sequence types of our strain collection. In addition, the six consecutive residues at amino acid position 426 to 431 found in *pbp2b* RFLP type 2 were previously described (25). Since these residues do not occur in other sequence types, they are presumably not critical to resistance development. Most of the PBP2x amino acid substitutions found in this study were also described previously (2, 17, 19). The amino acid substitution Leu546Val proceeding the KSG motif found in PBP2x of RFLP type 4 and RFLP types 1, 8 and 13 strains is presumed to be involved in cefotaxime resistance (2). All strains with this amino acid substitution had a reduced susceptibility for cefotaxime (MIC range 0.5 to 1 µg/ml; unpublished observations), confirming the importance of the Leu546Val substitution for cefotaxime resistance. Although the amino acid substitution Gln552Glu has also been observed in cefotaxime resistant isolates, the impact on resistance is unknown (17). Strains with the Gln552Glu substitution (RFLP types 6 and 31, RFLP type 7, RFLP type 20, and RFLP type 22) were all cefotaxime-susceptible (cefotaxime level: <0.016 to 0.032 µg/ml; unpublished observations), suggesting that the Gln552Glu does not play a role in cefotaxime resistance.

The *pbp* sequences identified in this study confirmed the acquisition of resistance by DNA transfer between non-pneumococcal species and pneumococci. Sequence blocks within *pbp1a*, *pbp2b* and *pbp2x* were identical to DNA from *S. mitis*, *S. oralis* and *S. sanguis*. Intra-

species horizontal transfer events among different genetic lineages of pneumococci have probably occurred as well, and this is especially suggested between strains with *pbp1a* RFLP types 1, 3, 6 and 10 and between strains with *pbp2b* RFLP types 5, 7 and 8. A recurrence of the recombination with viridans streptococci may have also taken place giving rise to a smaller section of the mosaic and is highly suggestive for *pbp2b* of the RFLP type 1B and 3C strains and for *pbp2x* RFLP type 4 and 7.

In conclusion, mutations in *pbp* sequences of most Dutch isolates were similar to those described in *pbp* sequences of strains isolated in other countries. This supports the hypothesis that multiple clones of penicillin-resistant pneumococci have been introduced and subsequently spread in The Netherlands as reported by Hermans *et al.* (16). However, novel combinations of sequence blocks in *pbp1a*, *pbp2b* and *pbp2x* were also identified, and suggests horizontal transfer of DNA between *pbp* genes of penicillin-resistant pneumococci previously described and *pbp* genes of susceptible strains.

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Chapter 6

Genetic relatedness within serotypes of penicillin-susceptible *Streptococcus pneumoniae* isolates

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SUMMARY

The molecular epidemiological characteristics of all *Streptococcus pneumoniae* strains isolated in a nation-wide manner from patients with meningitis in The Netherlands in 1994 were investigated. Restriction fragment end labeling analysis demonstrated 52% genetic clustering among these penicillin-susceptible strains, which is substantially lower than the percentage of clustering among Dutch penicillin-nonsusceptible isolates. Different serotypes were found within eight of the 28 genetic clusters, suggesting that horizontal transfer of capsular genes is common among the penicillin-susceptible strains. The degree of genetic clustering was much higher among serotype 3, 7F, 9V and 14 isolates than among isolates of other serotypes i.e. 6A, 6B, 18C, 19F and 23F. We further studied the molecular epidemiological characteristics of serotype 3 pneumococci, which are considered the most virulent serotype and is commonly associated with invasive disease in adults. Fifty epidemiologically unrelated penicillin-susceptible serotype 3 invasive isolates originating from the United States ($n = 27$), Thailand ($n = 9$), The Netherlands ($n = 8$) and Denmark ($n = 6$) were analyzed. The vast majority of the serotype 3 strains (76%) belonged to two genetically distinct clades that were observed in the United States, Denmark and The Netherlands. This indicates that two serotype 3 clones have independently disseminated in an international manner. Six serotype 3 strains were less than 85% genetically related to the other serotype 3 isolates. Our observations suggest that the latter isolates originate from horizontal transfer of the capsular type 3 gene locus to other pneumococcal genotypes. In conclusion, epidemiologically unrelated serotype 3 isolates were genetically more related than those of other serotypes. This observation suggests that serotype 3 has evolved only recently or has remained unchanged over large periods.

INTRODUCTION

Streptococcus pneumoniae continues to be a common cause of serious and life-threatening infections such as pneumonia, bacteremia and meningitis in both adults and children (1). Pneumococci can be classified according to differences in capsular polysaccharide structure. As many as 90 different capsular types can be divided by serotyping (11). The distribution of serotypes varies in different populations and different geographic areas, and certain pneumococcal serotypes are known to be more virulent than others (25, 29). Pneumococcal serotype 3 isolates are considered to represent the most virulent serotype. These isolates are

often responsible for invasive disease (18, 21), particularly in adults (16, 20). Bacteremia caused by this organism is considered to have the highest mortality rate as compared to the other serotypes (16, 21). To date, the frequency of penicillin resistance among serotype 3 isolates has remained low (17).

Serotyping as a tool for epidemiological studies has several disadvantages. *S. pneumoniae* is a naturally transformable species and frequent exchange of capsular genes occur (2-4, 14, 24). In addition, serotyping determines the variation in a single genetic locus, i.e. the *cps* locus. Therefore, several other typing methods have been developed to assist the identification of the relatedness between strains and their cellular structures. These methods include multilocus enzyme electrophoresis (MLEE) (10), penicillin-binding protein profile analysis (22, 23), pneumococcal surface protein A typing (23) and DNA fingerprint methods such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) (8), ribotyping, restriction fragment end labeling (RFEL) analysis, BOX PCR fingerprinting and DNA fingerprinting of the penicillin-binding protein (PBP) genes (15, 33). RFEL analysis provides a high degree of discriminatory power, and RFEL profiles are reproducible and suitable for computerized comparison (15). In addition, RFEL analysis provides a DNA fingerprint that represents multiple loci in the pneumococcal genome. This technique is routinely used in our laboratory to generate a data library of pneumococcal DNA fingerprints.

In this study, we investigated the molecular epidemiological characteristics of *S. pneumoniae* strains isolated in a nation-wide manner from patients with meningitis in The Netherlands in 1994. The genetic relatedness within pneumococcal serotypes was determined. In addition, we studied the molecular epidemiological characteristics of epidemiologically unrelated serotype 3 pneumococci from four distinct countries. The isolates were characterized by serotyping, RFEL analysis and PBP genotyping.

MATERIALS AND METHODS

Bacterial isolates. We studied a collection of *S. pneumoniae* strains (n = 153) isolated from Dutch patients suffering from meningitis in The Netherlands in 1994. These strains were collected by the National Reference Center for Bacterial Meningitis in a nation-wide manner and represent all pneumococcal meningitis isolates collected in a one-year period. In addition, these strains were penicillin-susceptible and are presumed to be epidemiologically unrelated. In addition, 42 penicillin-susceptible invasive serotype 3 pneumococci were isolated from patients in the United States (n = 27), Thailand (n = 9) and Denmark (n = 6). The latter strains

were also presumed to be epidemiologically nonrelated, since they were isolated from various geographic regions within these countries and at different time-points ranging from 1960 to 1962 and 1992 to 1998 (Table 1).

Serotyping. Pneumococci were serotyped on the basis of capsular swelling (quellung reaction) observed microscopically after suspension in antisera prepared at Statens Serum Institut Copenhagen, Denmark (9).

RFEL analysis. Typing of pneumococcal strains by RFEL analysis was performed as described by Van Steenbergen et al. (34) and adapted by Hermans et al. (15). Briefly, purified pneumococcal DNA was digested with the restriction enzyme *Eco*RI. The DNA restriction fragments were end labeled at 72°C with [α-32 P] dATP by using Taq DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The radiolabeled fragments were denatured and separated electrophoretically on a 6% polyacrylamide sequencing gel containing 8 M urea. The gel was transferred onto filter paper, vacuum dried (HBI, Saddlebrook, the United States), and exposed to ECL Hyperfilms (Amersham, Bucks, United Kingdom).

PBP genotyping. Genetic polymorphism of the penicillin resistance genes *pbp1a*, *pbp2b*, and *pbp2x* was investigated by restriction fragment length polymorphism (RFLP) analysis. PCR amplification of the PBP-encoding genes was performed in a 50-μl PCR buffer system containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% (wt/vol) Tween 20, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 10 pmol of the individual primers, 0.5 U of Taq DNA polymerase (Goldstar; Eurogentec), and 10 ng of purified chromosomal DNA. Cycling was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, the United States) and consisted of the following steps: predenaturation at 94°C for 1 min; 30 cycles of 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C; and a final extension at 72°C for 3 min. The primers used to amplify the genes *pbp1a*, *pbp2b*, and *pbp2x* were described previously (3, 6, 22). The amplification products (5 μl) were digested with the restriction endonuclease *Hin*fI and separated by electrophoresis in 2.5% agarose gels (28). Gels were scanned and printed with the Geldoc 2000 system (Biorad, Veenendaal, The Netherlands). The different PBP genotypes are represented by a three-number code (e.g., 06-14-43), referring to the RFLP patterns of the genes *pbp1a* [pattern 6], *pbp2b* [pattern 14], and *pbp2x* [pattern 43], respectively.

Table 1. Geographic origin and isolation date of the 50 penicillin-susceptible serotype 3 pneumococcal isolates.

strain code	original strain code	state, country	isolation date	RFEL-type	PBP type *(pbp1a-pbp2b-pbp2x)*
DK001	3-4 #484/61	Denmark	1961	294	02-02-71
DK002	3-7	Denmark	1961	294	02-02-71
DK003	3-1	Denmark	1960	292	02-02-71
DK004	785-91	Denmark	1991	167	02-02-71
DK005	59-92	Denmark	1992	299	02-02-71
DK006	3-6 #524/62	Denmark	1962	289	02-02-71
NL100	940099	The Netherlands	1994	167	02-02-71
NL101	940149	The Netherlands	1994	167	02-02-71
NL102	940275	The Netherlands	1994	167	02-02-71
NL103	940148.1	The Netherlands	1994	393	02-02-71
NL104	940723	The Netherlands	1994	300	02-02-71
NL106	941656.1	The Netherlands	1994	291	02-02-71
NL107	940081	The Netherlands	1994	290	02-02-71
NL108	941091	The Netherlands	1994	294	02-02-71
TH001	9	Thailand	1998	093	02-02-71
TH002	E10	Thailand	1998	096	09-02-71
TH003	15	Thailand	1998	122	02-02-03
TH004	14	Thailand	1998	122	02-02-03
TH005	E56	Thailand	1998	121	02-02-03
TH006	22	Thailand	1998	123	02-02-03
TH007	E89	Thailand	1998	165	02-02-71
TH008	26	Thailand	1998	105	02-02-03
TH009	59	Thailand	1998	242	02-02-71
US001	3-2 #2	USA	1960	294	02-02-71
US002	3-3 #Jersey	USA	1960	294	02-02-71
US003	4182-95	Alaska, USA	1995	295	02-02-71
US004	0205-94	Colorado, USA	1993	167	02-02-71
US005	0456-93	California, USA	1992	167	02-02-71
US006	0678-93	Alaska, USA	1993	167	02-02-71
US007	0680-93	Alaska, USA	1993	167	02-02-71
US008	0911-94	Alaska, USA	1993	167	02-02-71
US009	1807-92	Wisconsin, USA	1992	167	02-02-71
US010	1164-95	Pennsylvania, USA	1995	167	02-02-71
US011	1165-95	Pennsylvania, USA	1995	167	02-02-71
US012	1440-95	Maryland, USA	1995	167	02-02-71
US013	2197-93	Alaska, USA	1993	167	02-02-71
US014	2208-96	Oklahoma, USA	1996	167	02-02-71
US015	2280-93	Washington, USA	1993	167	02-02-71
US016	2341-95	Washington, USA	1995	167	02-02-71
US017	2564-93	Ohio, USA	1993	167	02-02-71
US018	A1	USA	1990s	167	02-02-71
US019	A2	USA	1990s	167	02-02-71
US020	A3	USA	1990s	167	02-02-71
US021	A5	USA	1990s	167	02-02-71
US022	A8	USA	1990s	167	02-02-71
US023	2926-94	Maryland, USA	1994	297	02-02-71
US024	A4	USA	1990s	297	02-02-71
US025	0934-94	California, USA	1994	298	02-02-71
US026	1603-92	Wisconsin, USA	1992	296	02-02-71
US027	1139-96	Oklahoma, USA	1996	076	02-02-71

Computer-assisted analysis of the DNA banding patterns. The RFEL types were analyzed with the Windows version of the Gelcompar software version 4 (Applied Maths, Kortrijk, Belgium) after imaging the RFEL autoradiograms with the Image Master DTS (Pharmacia Biotech, Uppsala, Sweden). The DNA fragments in the molecular size range of 160 to 400 bp were documented. The DNA banding patterns were normalized with pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages (27) and with the Jaccard similarity coefficient applied to peaks (32). Computer-assisted analysis and the methods and algorithms used in this study were according to the instructions of the manufacturer of Gelcompar. A tolerance of 1.2% in band positions was applied during comparison of the DNA patterns. For evaluation of the genetic relatedness of the isolates, we used the following definitions: (1) strains of a particular RFEL type are 100% identical on the basis of RFEL analysis (2) an RFEL cluster represents a group of RFEL types that differs only one band (approximately ≥95% genetic relatedness; (3) an RFEL clade represents a group of RFEL types that differs in less than 4 bands (approximately ≥85% genetic relatedness). The genetic heterogeneity is defined as the number of RFEL clades representing one or more strains divided by the total number of strains.

RESULTS

Epidemiology of invasive pneumococcal isolates in The Netherlands. The epidemiology of *S. pneumoniae* strains isolated in a nation-wide manner from patients suffering from meningitis in 1994 in The Netherlands was investigated. These strains (n = 153) were all found to be penicillin-susceptible, and were analyzed by serotyping, PBP typing and RFEL typing. The results are shown in Fig. 1 and Table 1. The invasive isolates represented 31 serotypes. The isolates represented the serotypes 1 (n = 3), 3 (n = 8), 4 (n = 3), 5 (n = 2), 6A (n = 7), 6B (n = 15), 7F (n = 7), 8 (n = 4), 9N (n = 4), 9V (n = 7), 10F (n = 2), 10A (n = 4), 11A (n = 2), 14 (n = 12), 15A (n = 1), 15C (n = 2), 16F (n = 2), 18F (n = 1), 18B (n = 2), 18C (n = 12), 19F (n = 18), 19A (n = 2), 22F (n = 1), 23F (n = 16), 23A (n = 1), 23B (n = 2), 24F (n = 3), 32A (n = 1), 33F (n = 5), 34 (n = 1) and 38 (n = 3).

Seven distinct PBP genotypes were observed displaying variation in RFLP patterns of *pbp2x* only. The PBP types 02-02-03, 02-02-71, and 02-02-02 occurred most frequently. In addition, all serotype 8 strains displayed PBP genotype 02-02-14, both serotype 5 strains displayed

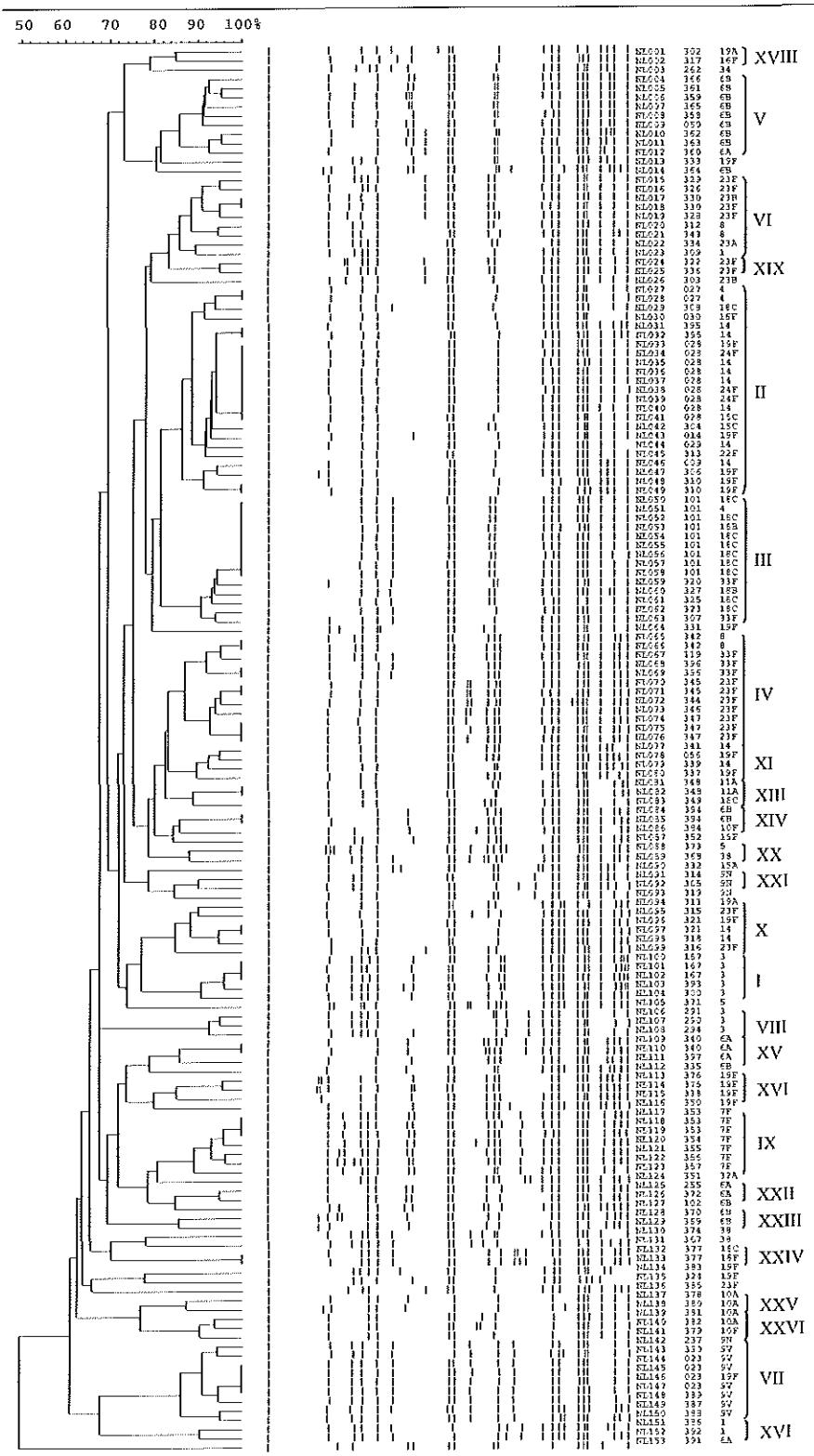
PBP genotype 02-02-15, and the single serotype 32A strain represented PBP genotype 02-02-16. Finally, three of the 18 serotype 19F strains displayed PBP genotype 02-02-05 (Table 2).

Table 2. PBP genotypes of the 153 *S. pneumoniae* strains isolated from patients with meningitis in 1994 in The Netherlands.

PBP genotype	number of strains	serotype of the strains
02-02-03	67	21 distinct serotypes
02-02-71	54	14 distinct serotypes
02-02-02	22	10 distinct serotypes
02-02-14	4	serotype 8
02-02-05	3	serotype 19F
02-02-15	2	serotype 5
02-02-16	1	serotype 32A

RFEL analysis divided the 153 strains into 116 distinct RFEL types. These RFEL types represented 28 genetic clusters, i.e. strains representing over 95% genetic relatedness, and 73 RFEL types that were less than 95% related to other strains. RFEL clusters were represented by 80 strains (52%). The cluster size varied from two (19 clusters) to nine strains (2 clusters). In addition, four clusters of three strains and three clusters of four strains were observed. The RFEL types 28 and 101 were the most predominant types. They were each represented by nine isolates. Within genetic clusters, different serotypes were observed. Eight of the 28 RFEL clusters displayed two or more serotypes (Table 3). The strain collection could be divided into 25 genetic clades, i.e. strains with more than 85% RFEL homology. The genetic clades varied in size from two to 23 strains (Fig. 1). Comparison of the penicillin-susceptible invasive isolates with penicillin-nonsusceptible strains representing 193 distinct RFEL types present in the international data library and representing 16 countries (14), revealed no overlap in RFEL types between penicillin-susceptible strains and penicillin-nonsusceptible strains.

Genetic relatedness within serotypes in The Netherlands. The genetic relatedness of strains within the nine most predominant serotypes present in the collection was investigated. The genetic heterogeneity is defined as the number of RFEL clades representing one or more strains divided by the total number of strains. The genetic heterogeneity within each serotype


50 60 70 80 90 100%
XVIII
V
VI
XIX
II
III
IV
XI
XIII
XIV
XX
XXI
X
I
VIII
XV
XVI
IX
XXII
XXIII
XXIV
XXV
XXVI
VII
XVI

was as follows: serotype 3 (2/8), 6A (4/7), 6B (6/15), 7F (1/7), 9V (1/7), 14 (3/12), 18C (4/12) 19F (11/18) and 23F (5/16). All strains of serotype 7F (n = 7) belonged to clade IX and all strains of serotype 9V (n = 7) belonged to clade VII. The strains of serotype 3 (n = 8) belonged to two distinct genetic clades, I and VIII. The strains of serotype 14 (n = 12) represented three distinct genetic clades, III, X and XI. Strains of serotypes 6B, 18C and 23F were genetically more heterogeneous, respectively. However, most strains of serotypes 6B, 18C and 23F belonged to one clade. Eight of the 15 serotype 6B strains belonged to clade V, nine of the 12 serotype 18C strains belonged to clade III and seven 7 of the 16 serotype 23F strains belonged to clade IV. Strains with the serotypes 6A and 19F displayed most heterogeneity in this collection of *S. pneumoniae* isolates, as seven serotype 6A strains were represented by four genetic clades and 18 serotype 19F strains were represented by 11 genetic clades (Fig. 1).

Table 3. RFEL clusters consisting of strains with different serotypes.

RFEL-cluster[a]	serotype
28	14 (n = 4), 15C (n = 1), 19F (n = 1), 24F (n = 3)
101	4 (n = 1), 18B (n = 1), 18C (n = 7)
23	9V (n = 3), 19F (n = 1)
328, 330	23F (n = 2), 23B (n = 1)
119, 342	8 (n = 2), 33F (n = 1)
56, 341	14 (n = 1), 19F (n = 1)
321	14 (n = 1), 19F (n = 1)
377	18F (n = 1), 18C (n = 1)

[a] For definition of RFEL cluster, see Materials and Methods section.

Genetic relatedness within serotype 3 isolates of distinct geographic origin. We investigated the molecular epidemiology of serotype 3 strains from The Netherlands (n = 8) and three additional countries: the United States (n = 27), Thailand (n = 9) and Denmark (n = 6). These fifty epidemiologically unrelated serotype 3 isolates were characterized by RFEL

Fig. 1. Genetic relatedness of 153 penicillin-susceptible invasive pneumococcal isolates based on the RFEL banding patterns of the isolates. The country code (NL, The Netherlands), strain codes, RFEL types and serotypes are depicted. Codes I to XI refer to genetic clades of pneumococcal strains (for definition see materials and methods section).

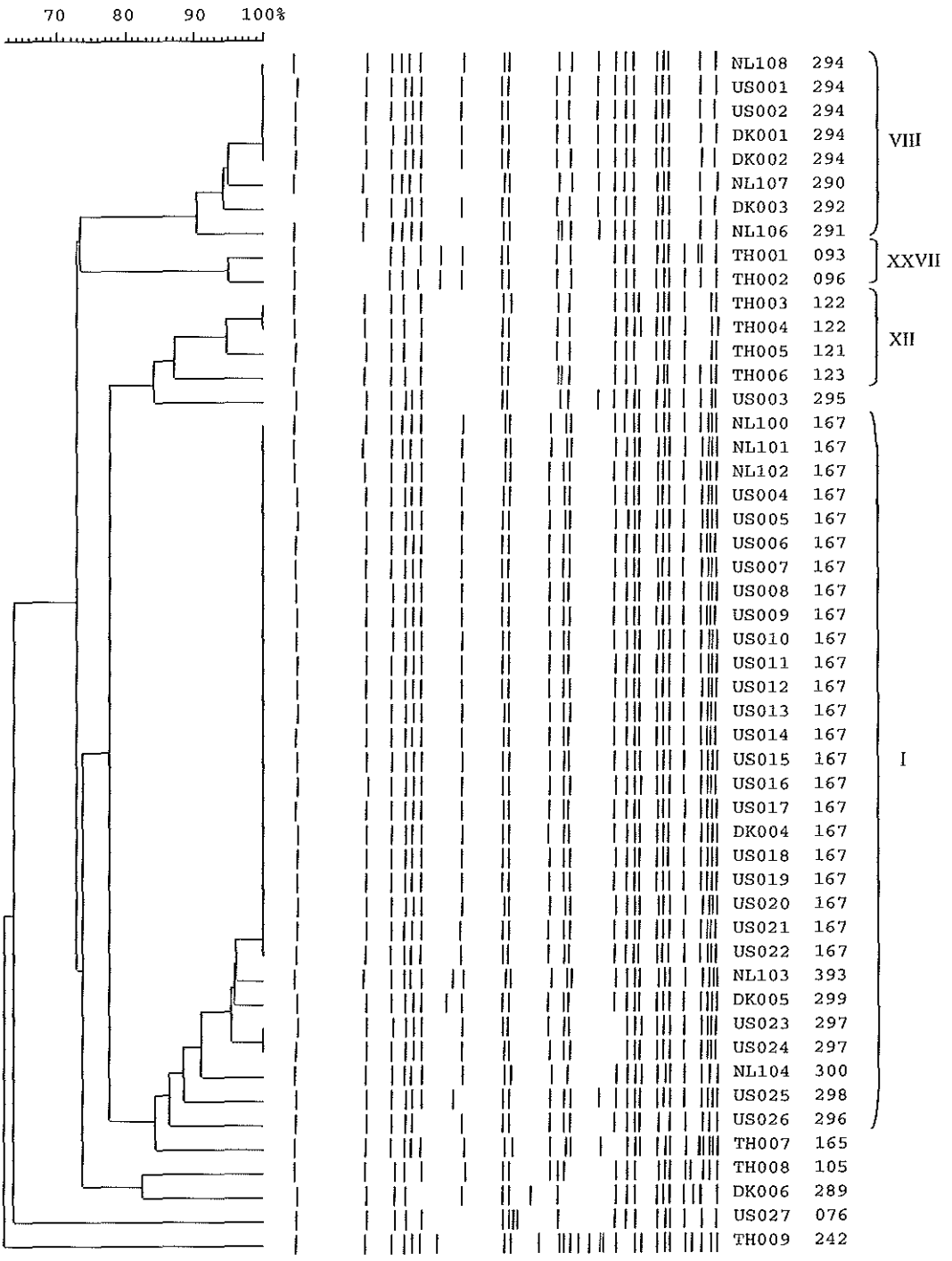
analysis. Four distinct RFEL clades and six RFEL types that were less than 85% related to other serotype 3 strains were observed among these strains (Fig. 2). The most predominant RFEL clade I represented 30 serotype 3 strains (60%). This RFEL clade was represented by 23 isolates from the United States, two isolates from Denmark and five isolates from The Netherlands. RFEL cluster VIII was represented by eight isolates (16%) and consisted of two American, three Danish and three Dutch strains. RFEL clade XII was represented by four Thai isolates. In addition, two Thai isolates formed a Thai-specific clade. Thus, 44 strains shared RFEL types with at least one other strain (88%). Six serotype 3 strains with RFEL types 295, 165, 105, 289, 76 and 242 did not match with the four genetic clades, and five of them did not match any of the 153 Dutch invasive isolates representing 116 RFEL types and 31 serotypes. In contrast, the serotype 3 strain with RFEL type 105 was genetically related (90.9%) with a serotype 19F strain representing RFEL type 352.

The serotype 3 collection was also analyzed by PBP typing. The PBP genotype 02-02-71 was invariably observed in the strains from the United States, Denmark and The Netherlands. The Thai strains displayed three distinct PBP genotypes: 02-02-03 (n = 5), 02-02-71 (n = 3), and 09-02-71 (n = 1) (Table 1).

DISCUSSION

Few studies have documented genotype analysis of penicillin-susceptible strains (13, 30), and of serotype specific strains (10, 19). We investigated the epidemiological characteristics of 153 penicillin-susceptible *S. pneumoniae* strains isolated from patients with meningitis in The Netherlands in 1994. The isolates represented 31 serotypes. The most predominant serotypes were 19F, 23F, 6B, 18C, 14, 3, 6A, 7F and 9V. Various investigators have reported the occurrence of horizontal transfer of capsular genes (2, 12-14). In the Dutch penicillin-susceptible isolates, horizontal transfer of capsular genes has occurred frequently. The high frequency of capsular exchange has been reported in molecular epidemiological studies of penicillin-resistant isolates from many countries (13, 14). This is the first study suggesting frequent occurrence of horizontal transfer of capsular genes among penicillin-susceptible strains.

Fig. 2. Genetic relatedness of 50 penicillin-susceptible pneumococcal serotype 3 isolates based on the RFEL banding patterns of the isolates. The country codes (NL, The Netherlands; US, the United States; DK, Denmark; TH, Thailand), strain codes and RFEL types are depicted. Codes I, VIII and XII refer to genetic clades of pneumococcal strains (for definition see Materials and Methods section).


70
80
90
100%
NL108 294
US001 294
US002 294
DK001 294
DK002 294
NL107 290
DK003 292
NL106 291
TH001 093
TH002 096
TH003 122
TH004 122
TH005 121
TH006 123
US003 295
NL100 167
NL101 167
NL102 167
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DK004 167
US018 167
US019 167
US020 167
US021 167
US022 167
NL103 393
DK005 299
US023 297
US024 297
NL104 300
US025 298
US026 296
TH007 165
TH008 105
DK006 289
US027 076
TH009 242
VIII
XXVII
XII
I

RFEL analysis revealed that 52% of the strains belonged to genetic clusters. The genetic clustering was substantially lower among the penicillin-susceptible isolates than among penicillin-nonsusceptible isolates in other studies (2, 5, 12-14, 26). Comparison of the penicillin-susceptible invasive isolates with 193 penicillin-nonsusceptible strains representing 193 distinct RFEL types present in the international data library and representing 16 countries revealed no overlap (13, 14).

The PBP genotypes 02-02-03, 02-02-71 and 02-02-02 were found most frequently. This observation corresponds with the PBP typing results of penicillin-susceptible pediatric carriage isolates in the American. population (31). Interestingly, four additional PBP genotypes (02-02-14, 02-02-15, 02-02-16 and 02-02-05) were identified which were present in the serotypes 8, 5, 32A and 19F, respectively. The serotype-specificity of the latter PBP genotypes suggests divergence of PBP genotypes before the origin of the capsular types 8, 5, 32A and 19F.

The genetic relatedness within the specific pneumococcal serotypes was highly variable. RFEL genotypes of serotype 6A and 19F strains displayed high levels of heterogeneity, i.e., strains of these serotypes represented many RFEL types that belong to many genetic clusters and genetic clades. In contrast, the RFEL genotypes within the serotype 7F, 9V, 14 and 3 strains were found genetically related. Interestingly and consistent with our observations, Canadian penicillin-susceptible isolates of serotype 3 and 7F were also more genetically related than isolates of other serotypes (19). Moreover, invasive penicillin-susceptible serotype 3 isolates from the United Kingdom also tend to be mutually more closely related than to isolates of other serotypes (10).

We focused on the molecular epidemiological characteristics of epidemiologically unrelated serotype 3 pneumococci and extended our serotype 3 collection with isolates from the United States, Thailand and Denmark. RFEL analysis demonstrated that serotype 3 strains isolated in these countries displayed a strong degree of genetic relatedness: the vast majority of the strains represented two distinct RFEL clades. Furthermore, both genetic clades harbored isolates from three countries: the United States, Denmark, and The Netherlands. These observations indicate that two serotype 3 clones have disseminated internationally. In addition, six Thai serotype 3 isolates belong to two RFEL clades (clade XII and XXVI). The data suggest strong genetic homogeneity within the serotype 3 pneumococci and support the observations in Canada and the United Kingdom (10, 19). Interestingly, the Canadian serotype 3 strains displayed two distinct genetic types, and the majority of the epidemiologically non-related serotype 3 strains from the United Kingdom grouped within

two genotypes. Moreover, MLST analysis of serotype 3 strains isolated in six distinct countries identified two major genetic clusters (7). Since the strains have been characterized with distinct typing methods, i.e. PFGE, MLEE, MLST and RFEL analysis and since there is no overlap in characterized strains, the genetic relatedness between the latter serotype 3 isolates and the strains characterized in this study is currently unknown. The remaining six serotype 3 RFEL types occurred once in our collection. Our observations suggest that these latter isolates have derived from horizontal transfer of the capsular type 3 gene locus to other pneumococcal genotypes.

PBP genotyping of the serotype 3 strains demonstrated limited variation in the *pbp1a*, *pbp2b* and *pbp2x* genes. All serotype 3 strains from the United States, Denmark and The Netherlands display PBP genotype 02-02-71. However, variation was demonstrated in the Thai serotype 3 isolates. The PBP type 09-02-71 was represented by a single Thai isolate. This PBP type was also specific for the penicillin-susceptible phenotype as there was no overlap with penicillin-nonsusceptible isolates from 16 different countries (14).

In conclusion, pneumococcal strains belonging to serotype 3 display limited genetic heterogeneity despite the lack of epidemiological relatedness. We hypothesize that this serotype has recently evolved or has remained unchanged for a prolonged period. The few serotype 3 isolates not belonging to the main clusters are presumably derived from horizontal transfer of capsular genes.

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Chapter 7

Immune-protective antibodies against capsular polysaccharides do not affect natural competence of *Streptococcus pneumoniae*: implications for current conjugate vaccination strategies?

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SUMMARY

We studied the effect of opsonisation of *Streptococcus pneumoniae* with capsular antibodies on horizontal transfer of DNA. Opsonisation did not inhibit DNA uptake. This suggests that horizontal transfer of capsular genes, which is an important escape mechanism of the pathogen, remains a potential threat for the efficacy of conjugate vaccination.

Streptococcus pneumoniae is an important human pathogen, which causes high morbidity and mortality. Genetic plasticity plays a central role in the biology of the pneumococcus. Horizontal gene transfer enables the bacterium to evolve rapidly by the acquisition of novel determinants and has resulted, under the selective pressure of host defense mechanisms and antibiotic treatment, in a great genetic variability of several virulence factors and resistance determinants (for review see references 2, 5, 10).

S. pneumoniae has the ability to express at least 90 unique capsular polysaccharide types (7, 19). Multidrug-resistant pneumococcal isolates are mostly associated with a limited number of serotypes (6). Pneumococci expressing the latter capsular types commonly cause disease and, therefore, attention is focussed on prevention of infection caused by these pneumococcal types (13). The current pneumococcal vaccine strategies concentrate on the use of conjugate vaccines, in which capsular polysaccharides are linked to a highly immunogenic carrier protein thereby switching the immune response against polysaccharides from T-cell independent to T-cell dependent. Consequently, the antibody response towards the polysaccharides is increased and a memory response is provided. However, the number of different capsular polysaccharide types that can be included in the vaccine is restricted (13). Several studies have shown that conjugate vaccination results in a change in serotype distribution of nasopharyngeal isolates from vaccine serotypes to non-vaccine serotypes (11; R. Dagan, N. Givon, P.Yagupsky, N. Porat, J. Janco, I. Chang, A. Kimura, J. Hackell, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother. abstr. 107-G, 1998). Such a shift may be enhanced by frequent horizontal exchange of capsular genes (1, 3, 4, 8, 12). Consequently, exchange of capsular genes in (multi)drug-resistant isolates from vaccine types to nonvaccine types is a potential threat to the efficacy of conjugate vaccines.

Increased expression of capsular polysaccharide results in a lower degree of competence (15, 18), whereby the capsule acts as a barrier that prevents competence factor from reaching its cellular target (20). Opsonisation may increase the steric hindrance by the capsule. In this study, we examined the effect of opsonisation with immune-protective capsular-type specific antibodies on pneumococcal competence.

For this purpose, the genetically unrelated clinical isolates of serotype 14 (strain NCTC 11902) and serotype 9V (strain 961729) were used. Bacteria were grown to an optical density at 600 nm = 0.1 at 37°C in Todd-Hewitt broth supplemented with 0.5% yeast extract (Difco laboratories, Detroit, USA) (THY broth) and for transformation experiments in THY broth with 0.16% bovine serum albumin and 0.01% $CaCl_2$ (competence broth). Bacterial pellets from 1 ml of culture were washed twice (5 min, 10,000 x g) with 1 ml of phosphate-buffered saline pH 7.5 (PBS), pre-heated at 37°C. The serum concentration that resulted in maximum opsonisation was determined by incubating the pellet for 10 min at 37°C in the presence of 0, 1.25, 2.5, 5 and 10% of capsular type 14 (batch HT95-0028 M091#7) and capsular type 9V (batch HT95-0022 M054#8) rabbit antiserum (Wyeth Lederle Vaccines, West Henrietta, United States), respectively, in 20 µl final volume while shaking. After washing twice with pre-heated PBS, the bacteria were incubated for 10 min at room temperature with 20 µl (1:5 dilution) of fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, United States) while shaking. The bacteria were washed twice and resuspended in 100 µl of 0.5% paraformaldehyde in PBS. The samples were analyzed in a flow cytometer (FACScan, Becton Dickinson, Mountain View, USA). The concentration of 10% capsular type 14 antiserum demonstrated the highest opsonic activity on type 14 pneumococci (Fig. 1) and, therefore, this serum concentration was used for further experiments. Similar data were observed with capsular type 9V antiserum and type 9V pneumococci (data not shown).

To investigate the effect of opsonisation on pneumococcal viability, the bacterial suspensions were opsonized with 10% serum as described above, washed twice, diluted and plated onto THY-agar plates (15 g of agar per liter) and incubated at 37°C with increased CO_2. The average number of viable type 14 and type 9V pneumococci before opsonisation was $4.37 \times 10^8 \pm 2.71 \times 10^8$ CFU/ml and $2.82 \times 10^8 \pm 4.37 \times 10^7$ CFU/ml, respectively, and after opsonisation with capsular-type specific antibodies $3.38 \times 10^8 \pm 7.42 \times 10^7$ CFU/ml and $2.75 \times 10^8 \pm 4.24 \times 10^7$ CFU/ml, respectively. Statistical analysis

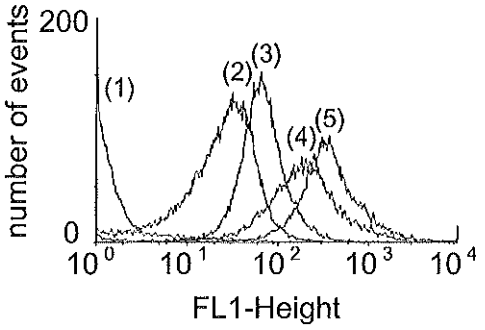

Fig. 1. The degree of pneumococcal serotype 14 opsonisation with increasing concentrations of immune-protective capsular type 14 specific hyperimmune rabbit antiserum as determined by FACS analysis. Y axis: number of pneumococci analyzed. X axis: the degree of immuno-fluorescence. Numbers indicate bacterial autofluorescence (1), specific binding with 1.25% (2), 2.5% (3), 5% (4) and 10% (5) of capsular type 14 antiserum.

(Student's T-test) showed no significant difference in bacterial viability before and after opsonisation.

In transformation experiments, pneumococci were opsonized with 10% serum, washed and resuspended in 1 ml of competence broth. The bacteria were transformed as described by Lacks (9) with DNA from streptomycin-resistant *S. pneumoniae* strain DP1617, which was the source for resistance marker DNA (16). The cells were incubated for 2.5 h with 0.4 and 1 µg of purified genomic DNA (17), respectively, and 300 ng of synthetic CSP-1 (14). The frequency of transformation of opsonized and nonopsonized pneumococci, respectively, was determined by comparing the number of colonies in the presence and in the absence of streptomycin (100 µg/ml). Statistical analysis using the Anova test demonstrated no differences in the uptake of marker DNA between pneumococci opsonized with immune-protective capsular-type specific antibodies and nonopsonized pneumococci for both pneumococcal clinical isolates (Fig. 2). In addition, different amounts of donor DNA (0.4 and 1 µg, respectively) did not affect transformation frequency (data not shown).

In this study, *in vitro* DNA transfer experiments demonstrated that pneumococcal opsonisation with immune-protective capsular-type specific antibodies did not affect the uptake of marker DNA. These data indicate that horizontal transfer of capsule loci is not

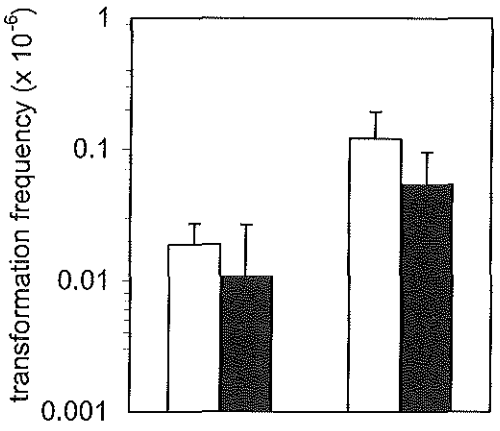

Fig. 2. Comparison of the genetic transformation frequencies of a type 14 and a type 9V clinical isolate, respectively, in the absence (open bars) and the presence (solid bars) of capsular-type specific antibodies. The transformation frequency (number of transformants/total number of bacteria) with resistance marker DNA was determined. Values are means of three independent determinations. Results are representative for two independent experiments.

inhibited in the presence of opsonizing antibodies and may provide an escape mechanism for pneumococci in vaccinated individuals. These recombination events have the potential to diminish the long-term effectiveness of current conjugate vaccination strategies, which contain a limited number of capsule types. Hence, molecular epidemiological studies are needed to monitor pneumococcal colonization and infection of individuals who will be vaccinated with conjugate vaccines.

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Chapter 8

Differential protein expression in phenotypic variants of *Streptococcus pneumoniae*

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SUMMARY

Streptococcus pneumoniae undergoes spontaneous phase variation resulting in opaque and transparent colony forms. Differences in colony opacity correlate with differences in virulence: the transparent variants are more capable of colonizing the nasopharynx, whereas the opaque variants show increased virulence during systemic infections. To gain insight into the pathogenesis of pneumococcal disease at the molecular level, protein expression patterns of the phenotypic variants of two pneumococcal strains were compared by high-resolution two-dimensional protein electrophoresis. In comparison with transparent variants, the opaque variants reduced the expression of two proteins and overexpressed one protein. The proteins were identified by mass spectrometric analysis. The protein overexpressed in the opaque phenotype revealed significant homology to elongation factor Ts of *Helicobacter pylori*. One of the two proteins that were underexpressed in the opaque variants revealed significant homology to the proteinase maturation protein PrtM of *Lactocobacillus paracasei*, a member of the family of peptidyl-prolyl *cis/trans* isomerases. A consensus lipoprotein signal sequence suggests that the putative proteinase maturation protein A, designated PpmA, is located at the surface of the pneumococcus and may play a role in the maturation of surface or secreted proteins. The second underexpressed protein was identified as pyruvate oxidase, SpxB. The lower SpxB expression in opaque variants most probably explains the reduced production of hydrogen peroxide, a reaction product of SpxB, in this variant. Since a *spxB*-defective pneumococcal mutant has decreased ability to colonize the nasopharynx (B. Spellerberg, D. R. Cundell, J. Sandros, B. J. Pearce, I. Indanpaan-Heikkila, C. Rosenow, and H. R. Masure. Mol. Microbiol. 19:803-813, 1996), our data suggest that SpxB plays an important role in enhancing the ability of transparent variants to efficiently colonize the nasopharynx.

INTRODUCTION

A critical process in the pathogenesis of infections caused by *Streptococcus pneumoniae* is the ability of the pathogen to adapt to various ecological niches in the human host. The pneumococcus colonizes the human nasopharynx and may spread locally to cause upper and lower respiratory tract infection. In some cases, pneumococci are able to enter the bloodstream and cause bacteremia or cross the blood-brain barrier and cause meningitis.

As is the case for other respiratory tract pathogens that frequently cause invasive infection, the ability to survive in these different host environments requires the regulation of the synthesis of key surface structures (10, 31). In *S. pneumoniae*, the reversible expression or phase variation in structures can be detected as spontaneous, reversible changes in colony morphology. Differences in surface molecules affect the arrangement of organisms within a colony, resulting in a change in colony appearance (32). The frequency of switching in colony morphology is highly variable from isolate to isolate and appears to be independent of in vitro growth conditions including pH, temperature and osmolarity (32). Genetic analysis has demonstrated that a stem-loop-forming repetitive element, BOX A-C, located upstream of the *glpF* gene, increases the variation in opacity (23).

Differences in colony morphology correlate with differences in in vitro and in vivo characteristics. In an infant rat model of nasopharyngeal carriage, only the transparent phenotype is able to establish dense and stable colonization of the mucosal surface of the nasopharynx (32). This can be explained by the enhanced binding of transparent pneumococci to buccal epithelial cells and their glycoconjugate receptors when compared to opaque pneumococci (5). Similarly, the adherence of transparent pneumococci to cytokine-stimulated human type II lung cells and human vascular endothelial cells is enhanced, as well as the receptors (*N*-acetyl-D-glucosamine and platelet-activating factor [PAF] receptor) that appear on these cells after cytokine stimulation (5). On the other hand, the opaque variant is more virulent in an animal model of systemic infection following intraperitoneal inoculation of adult mice (13). The higher virulence of the opaque variant in an in vivo model of sepsis correlates with decreased opsonophagocytic killing of opaque pneumococci in the in vitro phagocytosis assay (12). Finally, the transparent variants have an increased capacity to cross the blood-brain barrier (20). The higher binding affinity of transparent pneumococci to the PAF receptor on microvascular endothelial cells is suggested to result in increased transcytosis of bacteria across these cells.

To gain insight in the pathogenesis of pneumococcal disease at a molecular level, the relationship between several previously identified cell surface structures and opacity variation has been examined. The opaque phenotype is associated with larger amounts of capsular polysaccharide than is the transparent phenotype (12, 13). In contrast, the transparent phenotype produces increased amounts of teichoic acid, which contains phosphorylcholine (13, 30). Phosphorylcholine forms an anchor for at least eight choline-binding proteins (21). This structure is also part of PAF and is suggested to be important in the attachment to

cytokine-activated human cells via the PAF receptor by structural mimicry (5). Differences in the amount of phosphorylcholine might explain the switch between adherent and nonadherent phenotypes. Phenotypic variation also correlates with differential expression of cell surface proteins, including three choline binding proteins, LytA, PspA, and CbpA. Opaque variants undergo spontaneous lysis more slowly as a result of the decreased expression of the major amidase, LytA (33). In contrast to LytA, PspA is expressed in greater amounts in the opaque variant (13). PspA inhibits complement activation, thereby reducing the effectiveness of complement receptor-mediated pathways of clearance (27). In addition, PspA binds lactoferrin, an iron-sequestering glycoprotein that predominates in mucosal secretions, and may function in iron acquisition at mucosal surfaces (9). The differential expression of CbpA is similar to that of LytA, such that transparent variants express increased amounts of this protein (21). CbpA mediates adherence to cytokine-activated human lung epithelial and endothelial cells, is involved in invasion through microvascular endothelial cells, and participates in pneumococcal colonization of the nasopharynx (20, 21). The expression levels of LytA, PspA, and CbpA are unlikely to determine colony morphology directly, since mutants lacking each of the encoding genes still undergo variation in colony morphology (13, 21, 30).

The purpose of this study was to identify additional proteins that are differentially expressed in the phenotypic variants of *S. pneumoniae* to gain insight into the molecular changes that occur during phase variation. This information will contribute to an improved understanding of the molecular adaptation of phenotypic variants of pneumococci that result in changes in virulence and colony morphology. This paper reports on a comparison of expression patterns of the phenotypic variants of two pneumococcal strains by high-resolution two-dimensional protein electrophoresis and the identification of differentially expressed proteins by mass spectrometry.

MATERIALS AND METHODS

Bacterial strains, growth conditions and growth medium. Bacteria were removed from storage at -70°C and cultured at 37°C on Columbia agar supplemented with 5% defibrinated sheep blood (Oxoid, Basingstoke, United Kingdom) in an atmosphere of increased CO_2. Bacterial colonies were inoculated in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% yeast extract (Difco Laboratories) (THY broth) and grown at 37°C.

Broth cultures were plated onto THY agar plates (15 g of agar per liter) impregnated with 80 U catalase (Worthington Biochemical, Freehold, N.J.) per cm^2, and incubated at 37°C in an atmosphere of increased CO_2 that was provided in a candle extinction jar. Colony morphology of all cultures was determined as described previously (32). The pneumococcal strains used in this study are the opaque and transparent variants of type 9V clinical isolate P10 (32) and the opaque and transparent variants of type 6B clinical isolate P314 (12). *S. pneumoniae* D39 (2) and a *spxB*-defective mutant (D39, *spxB*::pHRM104, *phoA*$^+$; 26) were used as control strains used in the hydrogen peroxide assay. A *ppmA*-defective mutant (D39, *ppmA*::*ermAM*) (16a) was used as a control strain used for western blot analysis.

Protein sample preparation. The bacteria were cultured overnight in 10 ml of THY broth and subsequently to logarithmic growth phase (optical density at 550 nm = 0.3) in 250 ml of THY broth. This culture was harvested by centrifugation (1,500 x g for 15 minutes), and washed twice with 250 ml of phosphate-buffered saline (pH 7.5) and once with 10 ml of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA). The cells were disrupted by ultrasonic treatment (15 min with a microtip in a model 250 sonifier [Branson Ultrasonics, Danburry, Conn.]) while being held at 5°C. Protein concentrations were determined by the method of Bradford (3).

Two-dimensional protein gel electrophoresis, staining and computerized comparison of the protein profiles. Isoelectric focusing (pI 4 to 7) was performed with a Multiphor II electrophoresis unit and Immobiline DryStrips (Pharmacia Biotech, Uppsala, Sweden) as recommended by the manufacturers and includes the modifications described by Rabilloud et al. (18). The proteins were separated in the second dimension by gradient (12 to 20% polyacrylamide) polyacrylamide gel electrophoresis. Bacterial lysates containing 300 µg of protein were analyzed in the individual experiments. Polyacrylamide gels were stained with Coomassie brilliant blue (CBB) (24). The software program PD Quest (PDI, New York, N.Y.) was used for the computerized analysis of two-dimensional protein profiles. The relative amount of a protein, represented in parts per million (ppm), was determined by dividing the spot quantity by the total density of all proteins in the gel, thereby normalizing the amount of a single protein to the total amount of protein loaded. Significant differences in protein expression levels in the phenotypic variants were determined by the Mann-Whitney test with a set value of $P \leq 0.05$.

Purification, tryptic digest and mass spectrometric analysis of the proteins. The protein gel spots of interest were excised from the gel. The gel fragments were sliced thinly and washed twice for 15 min in 5% trichloroacetic acid (CCl_3COOH [Merck, Darmstadt, Germany]) and three times in distilled water. The gel fragments were equilibrated in sample buffer (pH 6.8) (0.1% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, 12 mM Tris-HCl, 0.01% bromophenol blue indicator [Merck]) for 1 h at room temperature. The proteins were concentrated by an agarose electrophoresis (1% agarose type VIII [Sigma, St. Louis, Mo.]) method as described by Rider et al. (19) and Gevaert et al. (7) on a model 150-A gel electrophoresis cell (Bio-Rad laboratories, Richmond, Calif.) with Pasteur pipettes. The agarose gel was stained with carconcarboxylic acid (Sigma), and the proteins were excised from the gel. The agarose fragments were washed with distilled water, and resuspended in 18 μl of digestion buffer (pH 8.0) (50 mM NH_4HCO_3, 5 mM $CaCl_2$). The agarose was melted at 85°C for 1 min. After it was cooled to 37°C 0.05 μg of trypsin (trypsin modified sequencing grade [Promega, Madison, Wis.]) per μl was added for at least 15 h at 37°C to digest the proteins. Trypsin was inactivated by adding 1 μl of 10% trifluoroacetic acid (CF_3COOH [Merck]). The tryptic digests were analyzed using a reversed phase microcapillary column switching high-pressure liquid chromatography system (16, 28). Peptide sequencing was performed on an LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, Calif.). Tandem mass spectrometric data were collected in data dependent scan mode for sequence information on single tryptic digest products. With Peptide Search (14), the deduced (partial) amino acid sequences were analyzed for matching sequences in all possible translation products of the most current version of the unfinished pneumococcal genome released by The Institute for Genomic Research (TIGR) (http://www.tigr.org/data/s_pneumoniae/) to identify the proteins. With the BLAST algorithm (1), putative pneumococcal proteins were analyzed for similarity to sequences deposited in the November 1999 version of the nonredundant protein database at the National Center for Biotechnology Information (Washington, D.C.).

Hydrogen peroxide assay. Hydrogen peroxide production by pneumococci was determined by the method of Pick and Keisari (17) and modified by Duane et al. (6). Bacteria were grown to mid-log phase, washed, and then grown in 250 μl of brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) in 96-well plates. Negative control wells contained 1000 U of catalase per ml. After 1 h of incubation at 37°C, the cultures were

harvested and centrifuged at 10,000 x g for 2 min, and the supernatant was filtered through 0.2-mm-pore-size filters. Phenol red and horseradish peroxidase were added to the assay buffer (5.0 mM K_2HPO_4, 1.0 mM KH_2PO_4, 140 mM NaCl, 0.5 mM glucose [pH 7.4]) at a final concentration of 0.46 mM and 0.046 U/ml, respectively, and the buffer was immediately used in the assay. Duplicate 250 μl aliquots of filtered supernatant were then mixed with 1.05 ml of assay mixture and incubated for 30 min at 37°C. The reactions were stopped by the addition of 5.0 μl of 1.0 N NaOH. The optical density was recorded at a 610 nm. Concentrations were calculated from a standard curve generated for each assay by adding known dilutions of 30% H_2O_2 to 250-μl aliquots of control supernatant. Control supernatant was heated to 100°C for 20 min before addition of H_2O_2 to eliminate catalase activity. Finally, the optical density of the culture as well as the number of CFU per milliliter was determined.

Western blot analysis. One-dimensional SDS polyacrylamide gel electrophoresis was carried out in the Bio-Rad minigel system with 13% polyacrylamide gels. Bacterial lysates (0.5 μg) were dissolved in sample buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 10 mM dithiothreitol, 10% glycerol, 0.01% bromophenol blue [Merck]), boiled for 5 min and subjected to electrophoresis (24). The proteins in the gel were transferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.) as described by Sambrook et al. (24). The membranes were washed twice for 10 min with Tris-HCl-buffered saline pH 7.5 supplemented with 0.05% Tween 20 (TBSt). The membranes were incubated with 2% bovine serum albumin for 1 h at room temperature, washed twice for 10 min each with TBSt, and incubated with 1:10,000 diluted anti-PpmA rabbit serum (16a) for at least 2 h with constant stirring. After the washing step, 1:4,000-diluted anti-rabbit immunoglobulin-alkaline phosphatase conjugate (Sigma) was added. After 1 h, the membranes were washed in TBSt and then in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). The blots were incubated with 0.33 mg of nitroblue tetrazolium (Sigma) per ml and 0.17 mg of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) per ml in alkaline phosphatase buffer in the dark. The staining reaction was stopped with distilled water.

RESULTS

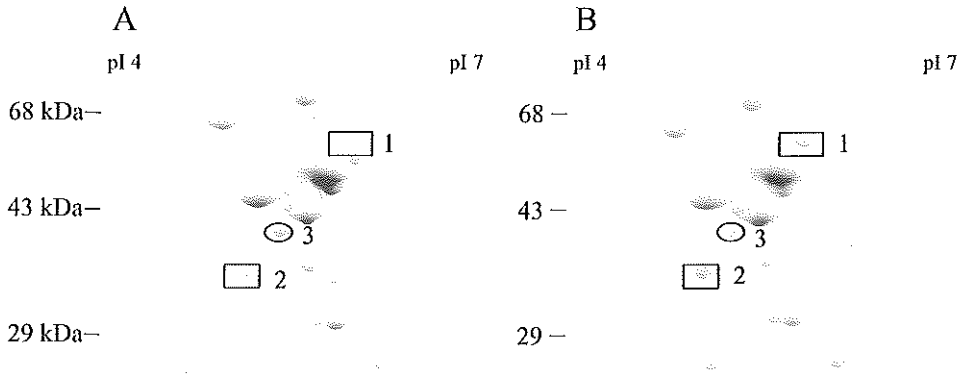

Fig. 1. Two-dimensional analysis of the cellular proteins of the opaque (A) and transparent (B) variants of pneumococcal clinical isolate p10. Bacterial proteins were separated by isoelectric focusing (pI 4 to 7) and gradient SDS-polyacrylamide gel electrophoresis (20 to 90 kDa). Circles and squares mark proteins with decreased and increased expression in the transparent variant, respectively, compared to the opaque variant. 1, 2, and 3 refer to the proteins discussed in the text.

Differentially expressed proteins in phenotypic variants of *S. pneumoniae*. The relative protein expression levels of phenotypic variants of strain P10 with opaque and transparent colony morphologies were compared using two-dimensional protein gel electrophoresis followed by computerized comparison of the CBB-stained gels. We performed this experiment with four cultures of opaque pneumococci and four cultures of transparent pneumococci. Approximately 200 distinct protein spots were analyzed. The quantity of two proteins, designated proteins 1 and 2, was decreased and the expression of protein 3 was increased in the opaque variant (Fig. 1). Protein 1 (65 kDa; pI 5.4) was clearly detectable in the CBB-stained protein expression profile of the transparent variant, but was not detectable in the opaque variant (Fig. 2). The quantity of protein 2 (35 kDa; pI 5.0) in the transparent variant was 2.6 times the quantity of this protein in the opaque variant. In contrast, the quantity of protein 3 (40 kDa; pI 5.1) was 2.1 fold greater in the opaque variant. The differences in the expression of proteins 1, 2, and 3 in the pneumococcal variants were statistically significant. The amounts of proteins 1, 2, and 3 were also compared in the phenotypic variants of clinical isolate strain P314 (serotype 6B). Again, proteins 1 and 2 were more prevalent in the transparent phenotype and protein 3 was more prevalent in the opaque phenotype; the levels of protein 1 and 2 were 1.3 times higher in the transparent variant and

that of protein 3 was 1.4 times higher in the opaque variant. Although the relative expression levels of all three proteins were less pronounced in the P314 variants, the trend in the differential expression of the proteins is comparable between strains P10 and P314 (Fig. 2).

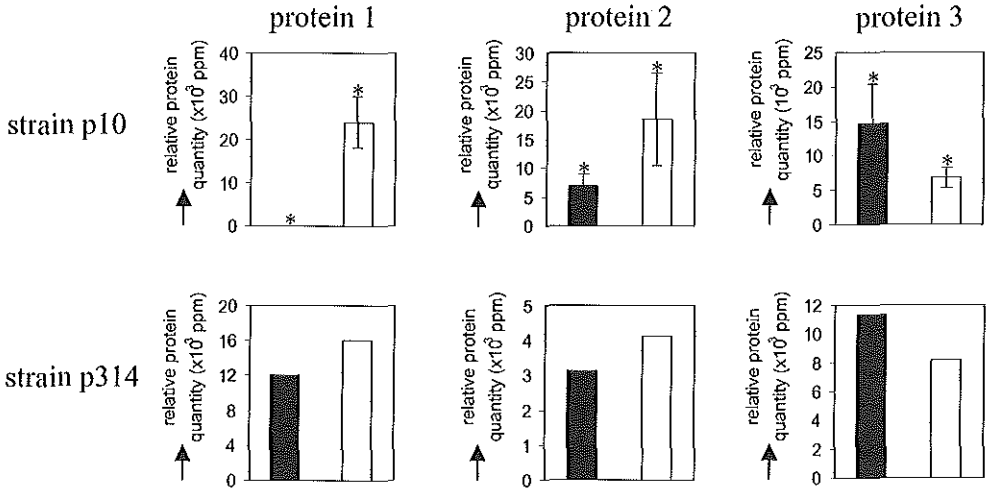

Fig.2. Relative expression levels of proteins 1, 2, and 3 as repeatedly observed in four independent experiments with the opaque variant (solid bars) and the transparent variant (open bars) of pneumococcal clinical isolate P10 (serotype 9V) and observed in a single experiment with clinical isolate P314 (serotype 6B). Asterisks depict significant difference ($P \leq 0.05$) in the relative protein quantity in the opaque and transparent variants.

The expression of pyruvate oxidase is increased in transparent pneumococcal variants. Tryptic digestion products of protein 1 were analyzed by mass spectrometry. Nine amino acid sequences were identical to the amino acid sequence of the pneumococcal pyruvate oxidase, SpxB, except for leucine 389, which was reported as a serine by Spellerberg et al. (26) (Genbank accession number L39074) and as an asparagine by TIGR (Fig. 3). Since Spellerberg et al. analyzed the nucleotide sequence of *spxB* of strain R6x (26) and TIGR sequenced the genome of a type 4 pneumococcal strain, this discrepancy may be a strain-specific difference. The calculated SpxB molecular mass of 65,183 Da correlated with the molecular ratio determined from two-dimensional protein electrophoresis, supporting the identity of the protein.

Since SpxB activity is known to result in the production of H_2O_2 (26), we measured H_2O_2 production in the phenotypic variants of strain P10. Opaque pneumococci produced <0.1 mmol of H_2O_2 per liter in 1 h, which is the detection limit of the assay, whereas transparent

pneumococci produced 0.6 mmol of H_2O_2 per liter in the same period (5 x 10^4 CFU/ml). In controls, strain D39 generated 0.45 mmol of H_2O_2 per liter under similar conditions and the SpxB mutant of D39 produced <0.1 mmol of H_2O_2 per liter. The higher production of H_2O_2 by the transparent variant correlates with the difference in the amount of protein observed by comparison of protein expression patterns.

001 MTQGKITASA AMLNVLKTWG VDTIYGIPSG TLSSLMDALA EDKDIRFLQV
051 RHEETGALAA VMQAKFGGSI GVAWGSGGPG ATHLINGVYD AAMDNTPFLA
101 ILGSRPVNEL NMDAFQELNQ NPMYNGIAVY NKRVAYAEQL PKVIDEACRA
151 AISKKGPAVV EIPVNFGFQE IDENSYYGSG SYERSFIAPA LNEVEIDKAV
201 **EILNNAERPV IYAGF**GGVKA GEVITELSRK IKAPIITTGK **NFEAFEWNYE**
251 **GLTSAY**RVGW KPANEVVFEA DTVLFLGSNF AFAEVYEAFK **NTEKFIQVDI**
301 **DPYK**LGKRH**A LDASILGDAG QAA**KAILDKV NPVESTPWWR ANVKNNQNWR
351 DYMNKLEGKT EGE**LQLYQVY NAINK**HADQD **AIYSLDVGS**T TQTSTRHLHM
401 TPKNMWRTSP LF**ATMGIALP** GGIAAKKDTP DRQVWNIMGD GAFNMCYPDV
451 ITNVQYDLPV INLVFSNAEY GFIKNKYEDT NKH**LFGVDFT** NADYGKIAEA
501 QGAVGFTVDR IEDIDAVVAE AVKLNKGGKT VVIDARITQH RPLPVEVLEL
551 DPKLHSEEAI KAFKEKYEAE ELVPFRLF**LE EEGL**QSRAIK

Fig.3. Amino acid sequence of the pneumococcal pyruvate oxidase (GenBank accession number L39074). The amino acid sequences of protein 1 derived by mass spectrometric analysis are depicted bold and underlined.

Increased expression of a proteinase maturation protein homologue in transparent pneumococcal variants. Mass spectrometric analysis of protein 2 resulted in seven peptides that were all identical to a putative translation product from the TIGR pneumococcal genome except for amino acid 142 (Fig. 4). This Tyr142-to-Gln/Lys substitution is most probably the result of strain-specific differences since TIGR sequenced the genome of a type 4 pneumococcal strain. Since glutamine and lysine have the same residue mass, it is not possible to distinguish between them by mass spectrometry. The open reading frame encoding a hypothetical protein of 322 amino acids is located at nucleotides 7632 to 8597 on contig 33 of the TIGR sequence. The calculated molecular mass of this protein (35.4 kDa) is similar to the molecular ratio of protein 2 (35 kDa) identified by two-dimensional protein electrophoresis, supporting the identity of the protein. We designated this pneumococcal protein PpmA (for "putative proteinase maturation protein A") because it showed significant homology to the proteinase maturation protein (PrtM) of *Lactobacillus paracasei* (SWISS-PROT accession number Q02473) (Fig. 4). PrtM is essential for the production of active

forms of the serine protease PrtP (8, 29). In *L. paracasei,* the gene encoding PrtP is located immediately downstream of *prtM* (11). In *S. pneumoniae,* no open reading frame homologous to serine proteases is found downstream of *ppmA*. PrtM is a membrane-bound lipoprotein in lactic acid bacteria (8). Also, *ppmA* contains a signal sequence of bacterial lipoproteins (Fig. 4).

```
Protein 2:  8    MKKKLLAGAITLLSVAT----LAACSKGSEGADLISMKGDVITEHQFYEQ
                 MKKK+     + L S AT     L+ C          + +  G  +TE  FY++
PrtM      : 1    MKKKMRL-KVLLASTATALLLLSGCQSNQADQKVATYSGGKVTESNFYKE

Protein 2: 54    VKSNPSAQQVLLNMTIQKVFEKQYGSELDDKEVDDTIAEEKKQYGENYQR
                 +K +P+ + +L NM I +       YG  +  K V+D      K+QYGEN+
PrtM      : 50   LKQSPTTKTMLANMLIYRALNHAYGKSVSTKTVNDAYDSYKQQYGENFDA

Protein 2: 104   VLSQAGMTLETRKAQIRTSKLVELAVKKVAEAELTDEAYKKAFDEYTPDV
                   LSQ G +  + K  +RT+ L E+A+KK+    ++++   K  +  Y P V
PrtM      : 100  FLSQNGFSRSSFKESLRTNFLSEVALKKL--KKVSESQLKAVWKTYQPKV

Protein 2: 154   TAQIIRLNNEDKAKEVLEKAKAEGADFAQLAKDNSTDEKTKENGGEITFD
                 T Q I L +++   + +    A G DFA LAK +S D  TK+NGG+I+F+
PrtM      : 148  TVQHI-LTSDEDTAKQVISDLAAGKDFATLAKTDSIDTATKDNGGKISFE

Protein 2: 204   SASTEVPEQVKKAAFALDVDGVSDVITATGTQAYSSQYYIVKLTKKTEKS
                 S +  +    K AA+ L     +              +            S
PrtM      : 197  SNNKTLDATFKDAAYKLKNGDYTQTPVKVTNGYEVIKMINHPAKGTFTSS

Protein 2: 254   SNIDDYKEKLKTVILTQKQNDSTFVQSIIGKELQAANIKVKDQAFQNIFT
                        K    +
PrtM      : 247  KKALTASVYAKWSRDSSIMQRVISQVLKNQHVTIKDKDLADALDSYKKPA

Protein 2: 304   QYI 306

PrtM      : 297  TTN 299
```

Fig. 4. The hypothetical pneumococcal protein encoded by nucleotide 7,632 to 8,597 on contig 33 of the TIGR pneumococcal genome (protein 2) displays 41% sequence similarity to the proteinase maturation protein (PrtM) of *L. paracasei* (SWISS-PROT accession number Q02473). The putative lipoprotein signal sequence is underlined. The amino acid sequences of protein 2 derived by mass spectrometric analysis are depicted in bold.

Differences in the amount of PpmA in the phenotypic variants were also demonstrated by Western blot analysis. Whole-cell lysates from a *ppmA*-negative mutant and its parent strain demonstrated the PpmA band position (Fig. 5, lanes 1 and 2). When equivalent amounts of bacterial lysates of phenotypic variants of strains P10 and P314 were compared on Western blots, the amount of PpmA was larger in the transparent variants (lanes 4 and 6).

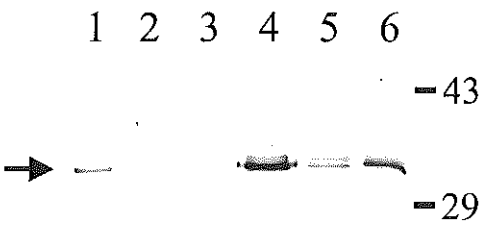

Fig. 5. Western blot analysis of phenotypic variants with anti-PpmA rabbit serum. The amount of PpmA was compared in equivalent amounts of whole cell lysates of opaque (lane 3 and 5) and transparent (lanes 4 and 6) variants of pneumococcal clinical isolate P10 (lanes 3 and 4) and P314 (lanes 5 and 6). The *ppmA*-deficient mutant (lane 2) and its parent strain D39 (lane 1) were used as controls. Numbers indicate the molecular size markers (in kilodaltons). The arrow indicates the PpmA protein band.

Reduced production of a putative elongation factor Ts in transparent pneumococcal variants. Mass spectrometric analysis of tryptic digestion products of protein 3 revealed three peptide sequences that were identical to a putative translation product from the TIGR pneumococcal genome (Fig. 6). The open reading frame encoding a hypothetical protein of 359 amino acids is located at nucleotides 911 to 1987 on contig 50 of *S. pneumoniae*. The calculated molecular mass (38.9 kDa) correlates with the molecular ratio of protein 3 (40 kDa) determined by two-dimensional protein electrophoresis. This protein showed significant homology to elongation factor Ts (EF-Ts) of *Helicobacter pylori* J99 (SWISS-PROT accession number AE001567) (Fig. 6).

DISCUSSION

Phenotypic variation is an important mechanism that allows bacterial pathogens to adapt to different host environments. The ability to survive in different host environments is the result of molecular adaptation, e.g., changes in the expression of specific cell surface components (10, 31). The expression of four previously identified proteins has been examined in phase variants of *S. pneumoniae*. PspA is expressed in larger amounts in the opaque phenotype, whereas CbpA and LytA are expressed in larger amounts in the transparent phenotype (13, 21, 33). No difference in pneumolysin expression has been found by Western blot analysis of whole cell lysates of the opaque and transparent variants (13). In this study, we identified and characterized three additional proteins that are differentially expressed in phenotypic variants

```
Protein 3: 14  MAEITAKLVKELREKSGAGVMDAKKALVETDGDIEKAIELLREKGMAKAA
               M+ I+A+LVK+LR+ + AG+MD KKALVE  GD++KAI+ LREKG++KAA
EF-Ts    : 1   MSGISAQLVKKLRDLTDAGMMDCKKALVEVAGDLQKAIDFLREKGLSKAA

Protein 3: 64  KKADRVAAEGLTGVYVNGNV--AAVIEVNAETDFVAKNAQFVELVNTTAK
               KKADR+AAEG+  + V  +   A ++E+N+ETDFVAKN  F ELV  T +
EF-Ts    : 51  KKADRIAAEGVVALEVAPDFKSAMMVEINSETDFVAKNEGFKELVKKTLE

Protein 3: 112 VIAEGKPANNEEALALIMPSGETLEAAYVSATATIGEKISFRRFALIEKT
                I        EE L   + + +  E    S  A IGE I  R+ A ++
EF-Ts    : 101 TIKTHNIHTTEELLKSPLDN-KPFEEYLHSQIAVIGENILVRKIAHLKAP

Protein 3: 162 DAQHFGAYQHNGGRIGVISVVEGGDEA-------LAKQLSMHIAAMKPTV
                      +Y H+  R+GV+  +E  +E         LA+ ++MH AAMKP V
EF-Ts    : 151 SSHIINGYAHSNARVGVLIAIEYNNEKNAPKVVELARNIAMHAAAMKPQV

Protein 3: 205 LSYKELDEQFVKDELAQLNHVIDQDNESRAMVNKPALPHLKYGSKAQLTD
               L  K+    FVK E   L   I++DNE    + KP       +GS+ +L+D
EF-Ts    : 201 LDCKDFSLDFVKKETLALIAEIEKDNEEAKRLGKPLKNIPTFGSRIELSD

Protein 3: 255 DVIAQAEADIKAELAAEGKPEKIWDKIIPGKMDRFMLDNTKVDQAYTLLA
               +V+A  +   + EL  +GKPEKIWDKI+PGKM+RF+ DNT +DQ  TLL
EF-Ts    : 251 EVLAHQKKAFEDELKEQGKPEKIWDKIVPGKMERFIADNTLIDQRLTLLG

Protein 3: 305 QVYIMDDSKTVEAYLESV------NASVVEFARFEVGEGIEKAANDF
               Q Y+MDD KT+   +          N  + E+ RFE+GEGIEK   +F
EF-Ts    : 301 QFYVMDDKKTIAQVIADCSKEWDDNLKITEYVRFELGEGIEKKTENF
```

Fig. 6. The hypothetical pneumococcal protein encoded by nucleotides 911 to 1987 on contig 50 of the TIGR pneumococcal genome (protein 3) displays 61% sequence similarity to EF-Ts of *H. pylori* J99 (SWISS-PROT accession number AE001567). The amino acid sequences of protein 3 derived by mass spectrometric analysis are depicted in bold.

of *S. pneumoniae*. The minimal protein amount necessary for detection and identification by mass spectrometric analysis is about 500 fmol. For this reason, gels were stained with CBB and not by the more sensitive silver-stain method, since only proteins visualized by CBB staining reached the mass spectrometry analysis detection level. This may explain why we did not confirm the diffential expression of LytA, PspA, and CbpA in the phenotypic variants and why we identified differential expression of three proteins only.

We demonstrated that the expression of pyruvate oxidase was increased in the transparent variants. Pyruvate oxidases are crucial for aerobic carbohydrate metabolism of several streptococci (4). This enzyme decarboxylates pyruvate to acetyl phosphate, resulting in the release of H_2O_2 and CO_2. In *S. pneumoniae,* the release of H_2O_2 is mainly the result of SpxB activity, since a *spxB*-defective mutant produces virtually no H_2O_2 in comparison with the parent strain (26). Therefore, the higher expression of SpxB in the transparent phenotype most probably explains the increased production of hydrogen peroxide. Spellerberg et al. have identified pyruvate oxidase as an indirect determinant of virulence in *S. pneumoniae*

(26). A *spxB* mutant has a decreased ability to colonize the nasopharynx in a rabbit model. This is reflected by the lower capacity of binding to the nasopharyngeal cells and to their glycoconjugate receptors. The decreased ability of *spxB*-defective pneumococcal mutants to colonize the nasopharynx and the increased expression of SpxB in transparent variants suggests the potentially important role of SpxB in efficient colonization of the nasopharynx by transparent variants (26). The expression level of SpxB is unlikely to directly determine colony morphology, since the *spxB* mutant of D39 still varies in colony morphology (J. N. Weiser, unpublished data).

The second protein with increased expression in the transparent variant demonstrated significant sequence homology to proteinase maturation protein (PrtM) of *L. paracasei*. *L. paracasei* and other lactic acid bacteria are used in the food industry for the production of a variety of fermented milk products. During growth, these bacteria produce cell envelope-located serine proteases that break down caseins, the major proteins in milk. PrtM is a *trans*-acting protein involved in the maturation (processing into active proteins) of serine protease, PrtP (29). PrtM belongs to the family of peptidyl-prolyl *cis/trans* isomerases that are thought to assist in protein folding by catalyzing the *cis-trans* isomerization of the peptidyl-prolyl bonds in peptides and proteins (22). PpmA contains an N-terminal lipoprotein signal sequence, which suggests that PpmA, like PrtM (8), is membrane bound. We hypothesize that PpmA also functions as a membrane-bound molecular chaperone. In *L. paracasei*, the gene encoding PrtP is located immediately downstream of *prtM* (11). Both *prtM* and *prtP* were found to be transcribed from the same promoter region but in opposite directions (29). In *S. pneumoniae*, no open reading frame homologous to a serine protease could be located in the direct vicinity of *ppmA*. The pneumococcal proteins activated by PpmA are currently unknown. We demonstrated differential expression of PpmA in the phenotypic variants of *S. pneumoniae*, which suggests that PpmA may play a role in the pathogenesis of infections. PpmA is more prevalent in the transparent phenotype that is selected for during nasopharyngeal colonization, suggesting that PpmA is directly involved in adherence through maturation of surface proteins with adherence properties or indirectly by the activation of proteases or other secreted proteins.

The expression of the third protein, identified as EF-Ts was increased in the opaque phenotype. EF-Ts is essential for the elongation of the polypeptide chain during protein synthesis. The protein mediates the regeneration of EF-Tu•GDP into the active form EF-Tu•GTP. This active form of EF-Tu facilitates the entry of aminoacyl-tRNA to the ribosome,

enabling protein synthesis. Differential expression of EF-Ts has been found previously in the gram-negative bacterium *Coxiella burnetii* (25), the causative agent of Q fever. This obligate intracellular parasite replicates in distinct morphological forms that may allow potential life cycle variants to survive the harsh environment of the phagolysosome. Two distinct morphological forms of *C. burnetii* have been described, a large-cell variant and small-cell variant. Large-cell variants are metabolically more active than small-cell variants (15). This is supported by data showing that EF-Ts and EF-Tu were more prevalent in large-cell variants (25). We hypothesize that the increased presence of elongation factor Ts in the opaque variants of *S. pneumoniae* indicates that, like in *C. burnetii*, the opaque variants are metabolically more active, which may explain the rapid invasive growth characteristics of these variants.

This study shows that the combination of two-dimensional protein gel electrophoresis, mass spectrometry, and genomics is a powerful tool for the identification of differentially expressed proteins in phenotypic variants of *S. pneumoniae*. We have identified differential expression of pyruvate oxidase, a new member of the family of peptidyl-prolyl *cis/trans* isomerases (PpmA), and EF-Ts in pneumococcal phenotypic variants of type 9V and 6B clinical isolates. We hypothesize that the higher expression of both pyruvate oxidase and PpmA in the transparent phenotype correlates with increased adhesive properties and ability to colonize the nasopharynx. The higher expression of EF-Ts in the opaque variant might indicate a higher metabolic activity.

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Chapter 9

Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract

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SUMMARY

An inverse correlation between colonization of the human nasopharynx by *Streptococcus pneumoniae* and *Haemophilus influenzae*, both common upper respiratory pathogens, has been reported. Studies were undertaken to determine if either of these organisms produces substances which inhibit growth of the other. Culture supernatants from *S. pneumoniae* inhibited growth of *H. influenzae*, whereas culture supernatants from *H. influenzae* had no effect on the growth of *S. pneumoniae*. Moreover, coculture of *S. pneumoniae* and *H. influenzae* led to a rapid decrease in viable counts of *H. influenzae*. The addition of purified catalase prevented killing of *H. influenzae* in coculture experiments, suggesting that hydrogen peroxide may be responsible for this bactericidal activity. *H. influenzae* was killed by concentrations of hydrogen peroxide similar to that produced by *S. pneumoniae*. Hydrogen peroxide is produced by the pneumococcus through the action of pyruvate oxidase (SpxB) under conditions of aerobic growth. Both an *spxB* mutant and a naturally occurring variant of *S. pneumoniae*, which is downregulated in SpxB expression, were unable to kill *H. influenzae*. A catalase-reversible inhibitory effect of *S. pneumoniae* on the growth of the respiratory tract pathogens *Moraxella catarrhalis* and *Neisseria meningitidis* was also observed. Elevated hydrogen peroxide production, therefore, may be a means by which *S. pneumoniae* is able to inhibit a variety of competing organisms in the aerobic environment of the upper respiratory tract.

INTRODUCTION

Bacterial pathogens are generally studied individually, although in their natural environment they often coexist or compete with multiple other microbial species. The focus of this report is bacterial pathogens that commonly colonize and infect the respiratory tract of humans. The results of clinical studies that surveyed the etiologic agents in cases of otitis media in children and chronic bronchitis in adults showed that *Streptococcus pneumoniae* and *Haemophilus influenzae* are the most prevalent bacterial pathogens (14, 23). The frequency with which these two species are isolated from the same specimen, however, is significantly less than would be predicted based on their relative prevalence (25, 30). This suggests that there may be inhibitory effects of one species on the other in vivo. This would not be an unexpected finding considering our current understanding of the pathogenesis of colonization and infection by these species. For instance, since both *S. pneumoniae* and *H. influenzae* express

cell surface phosphorylcholine, which mediates adherence to the receptor for platelet-activating factor, there may be competition for the same host cell receptor (12, 35, 46). In addition, phosphorylcholine is immunogenic, and antibody generated against phosphorylcholine from one species may promote clearance of a heterologous species bearing the same epitope (9, 31, 45). However, the presence of phosphorylcholine is required for viability in the case of the pneumococcus, while *H. influenzae* is able to switch off expression of this antigen (44, 51). Another example is the neuraminidase secreted by the pneumococcus, which has the potential to remove sialic acid residues from bacterial competitors known to express this as a cell surface structure (6, 10). The lipopolysaccharide of the respiratory tract pathogen, *Neisseria meningitidis*, and at least some strains of *H. influenzae* are sialylated and, in the case of the former, this modification acts to increase resistance to clearance mediated by complement (17, 21, 28, 29).

In order to begin to examine the interactions of the coinhabitants of the heavily colonized mucosal surface of the human upper respiratory tract, we tested the effect of coculture in vitro on growth and viability. These studies revealed that the pneumococcus produces an inhibitory substance that was shown to be hydrogen peroxide. This suggests that the production of H_2O_2 by *S. pneumoniae*, previously shown to be cytotoxic for cultured alveolar epithelial cells, may also be an effective mechanism for limiting or eliminating competitive flora, including common pathogens such as *H. influenzae* and *N. meningitidis*, which share the same microenvironment (15). These species, furthermore, are sensitive to levels of peroxide generated by the pneumococcus despite their production of catalase, an enzyme that acts to eliminate hydrogen peroxide (7, 8, 37).

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Strains used in this study are described in Table 1. All strains were cultured in brain heart infusion broth (BHI) with or without 1.5% agar (Difco Laboratories, Detroit, Mich.). *H. influenzae* was grown in BHI medium supplemented with hemin and L-histidine (dissolved in 1% triethanolamine, each at a final concentration of 2.5 μg/ml) (sBHI) plus NAD (2.0 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). All organisms were grown at 37°C with aeration except streptococci, which were grown without shaking. Plates containing streptococci and neisseriae were incubated in the presence of supplemental

carbon dioxide using candle extinction jars. Pneumococci were plated on BHI containing 200 U of bovine liver catalase per ml (Worthington Biochemical, Freehold, N.J.).

Supernatant inhibition assays. Cultures of *S. pneumoniae* P394 were grown in liquid BHI medium at 37°C under atmospheric conditions. After reaching mid-log phase (optical density at 620 nm [OD_{620}] = 0.3 to 0.4), the cultures were harvested and spun at 10,000 x *g* for 2 min, and the supernatant was filtered through 0.2-mm (pore-size) filters. The target organism was grown in liquid BHI or sBHI medium to mid-log phase (OD_{620} = 0.3 to 0.4) and then diluted 10-fold in phosphate-buffered saline (PBS). Bacterial lawns were obtained by spreading 50 μl of diluted culture on BHI or sBHI agar with or without 200 U of catalase per ml. Then, 10-μl aliquots of supernatant were spotted onto these plates and allowed to dry prior to incubation at 37°C for 16 h. In some experiments, aliquots of supernatant were treated with proteinase K (final concentration, 50 μg/ml; Sigma) at 37°C for 1 h or heated to 65°C for 20 min prior to adding them to plates containing target organisms.

Coculture experiments. Bacteria were grown in BHI medium at 37°C until mid-log phase (OD_{620} = 0.3 to 0.4), centrifuged for 2 min at 10,000 x *g* and 4°C, washed in ice-cold Hanks balanced saline solution (HBSS; Gibco BRL, Gaithersburg, Md.), and then resuspended in BHI at the original culture volume. Equal volumes of *S. pneumoniae* and the target strain were then mixed and incubated at 37°C in 96-well polystyrene microtiter plates (Dynex Technologies, Inc., Chantilly, Va.). As a negative control, each strain was mixed with an equal amount of BHI alone. Where indicated, individual wells were supplemented with catalase (final concentration, 1,000 U/ml). Serial dilutions were then prepared in HBSS, and an aliquot was plated on BHI agar plates containing catalase (final concentration, 200 U/ml) for viable counts. Dilutions of mixed cultures were spread on BHI plates supplemented with 2.0% Fildes enrichment (Difco) and grown under atmospheric conditions which selectively inhibited the growth of *S. pneumoniae* and allowed enumeration of the target species. Removal of the Fildes enrichment, which provides a source of hemin and NAD, provided selective conditions preventing the growth of *H. influenzae*.

Hydrogen peroxide sensitivity assays. Bacteria were grown in BHI medium at 37°C until mid-log phase (OD_{620} = 0.3 to 0.4), centrifuged for 2 min at 10,000 x *g* and 4°C, washed in ice-cold HBSS, and resuspended in fresh BHI medium. Resuspended bacteria were added to

Table 1. Hydrogen peroxide sensitivity and production by various bacterial pathogens

Species	MIC (mM)[a]	MBC (mM)[b]	H_2O_2 generated (mM)[c]	Source or reference [d]
Gram-negative				
H. influenzae Rd	0.4	0.5	<0.1	26
H. influenzae Eagan	0.4	0.5	<0.1	26
N. meningitidis MC58C3	0.4	5	<0.1	32
M. catarrhalis Bc1	1.1	160	<0.1	Clinical isolate
E. coli RS218	ND	15	<0.1	1
S. enterica serovar Typhimurium LT2	ND	20	<0.1	Collection of K. Sanderson
K. pneumoniae Kp1	ND	20	<0.1	Clinical isolate
P. aeruginosa PA01	ND	60	<0.1	ATCC 15692
Gram-positive				
S. pyogenes P87	ND	40	<0.1	Clinical isolate
S. agalactiae P60	ND	80	<0.1	Clinical isolate
S. equisimilis P107	ND	20	<0.1	Clinical isolate
E. faecium P119	ND	80	<0.1	Clinical isolate
S. aureus A1	ND	10	<0.1	Clinical isolate
S. pneumoniae strains				
P394 (type 4)	1.6	80		TIGR genome strain
D39 (type 2)	1.2	80	0.44 ± 0.08	4
P383 (type 6B)	ND	ND	0.53 ± 0.08	22
P384 (type 6A)	ND	ND	0.71 ± 0.13	22
P878 D39 (*spxB*::Tn*phoA*)	1.6	80	<0.1	38
P62 (type 9V opaque variant)	ND	ND	<0.1	22
P64 (type 9V transparent variant)	ND	ND	0.43 ± 0.13	22

[a]The MIC was determined as the minimum concentration of H_2O_2 necessary to prevent turbid growth of a 1-in-50 inoculum of a stationary-phase culture following overnight incubation at 37°C. ND, not determined.
[b]The MBC was determined as the minimum concentration of H_2O_2 necessary for >99.9% killing of washed, log-phase cells in BHI medium after 30 min at 37°C.
[c]H_2O_2 concentration present in culture supernatants after incubating approximately 5 x 10^7 washed, log-phase cells for 1 h in BHI medium at 37°C.
[d]TIGR, The Institute for Genome Research.

microtiter plate wells in duplicate containing twofold dilutions of H_2O_2 (Sigma) in BHI medium and incubated at 37°C for 30 min. Aliquots from each well were applied to BHI agar plates containing 200 U of catalase per ml for viable counts. The concentration of H_2O_2 required to cause a 99.9% decrease in the number of colonies compared to the negative control without peroxide was recorded as the minimum bactericidal concentration (MBC). For MIC determination, 50-fold dilutions of stationary-phase cultures in BHI containing twofold dilutions of H_2O_2 were incubated at 37°C overnight. The minimum concentration necessary to prevent turbid growth was considered the MIC.

Hydrogen peroxide production assays. Hydrogen peroxide production was measured in an assay developed by Pick and Keisari and modified by Duane and coworkers (15, 36).

Bacteria were grown in BHI medium at 37°C until mid-log phase (OD_{620} = 0.3 to 0.4), centrifuged for 2 min at 10,000 x *g* and 4°C, washed in ice-cold HBSS, and resuspended in BHI medium to twice the original culture volume. Wells for negative controls contained 1,000 U of catalase per ml. After 1 h of incubation under atmospheric conditions at 37°C, the cultures were harvested, spun at 10,000 x *g* for 2 min, and filtered through a 0.2-μm (pore-size) membrane. Immediately prior to the assay, phenol red and horseradish peroxidase were added to peroxide assay buffer (5.0 mM K_2HPO_4, 1.0 mM KH_2PO_4, 140 mM NaCl, 0.5 mM glucose; pH 7.4) at final concentrations of 0.46 mM and 0.046 U/ml, respectively. Aliquots of filtered supernatant were added to the assay mixture at a ratio of 1 to 4 and incubated for 30 min at 37°C in duplicate. After the reactions were stopped by the addition of NaOH (final concentration, 0.004 N) the absorbance was recorded at a wavelength of 610 nm. Concentrations were calculated in comparison to a standard curve with known amounts of H_2O_2 added to control supernatant from wells containing catalase which had been heated to 100°C for 20 min to eliminate catalase activity.

Two-dimensional protein gel electrophoresis. Two-dimensional protein gel electrophoresis followed by staining, computerized comparison, and mass spectrometric analysis of the proteins, was done as described elsewhere (35a).

Western transfer and immunoblotting. P878 containing an in-frame fusion of Tn*phoA* to the gene for pyruvate oxidase (*spxB*) was grown on tryptic soy agar plates containing catalase (200 U/ml) (38). Bacteria were grown for 16 h at 37°C under atmospheric conditions (20% O_2, 0.03% CO_2), in a candle extinction jar (17% O_2, 3%CO_2), or in the GasPak anaerobic system (<0.01% O_2, 10% CO_2) (Becton Dickinson, Cockeysville, Md.). Cells were harvested from plates, adjusted to equal density based on absorbance at 620 nm, washed in cold PBS, and treated at 100°C for 5 min in gel loading buffer (50 mM Tris-Cl, pH 6.8; 100 mM β-mercaptoethanol; 10% glycerol; 2% sodium dodecyl sulfate [SDS], 1% bromophenol blue) prior to separation in SDS–10% polyacrylamide gel electrophoresis (PAGE) gels. Equal loading was confirmed by measurement of total protein in whole-cell sonicates using the Micro BCA Protein Assay (Pierce Chemical Co., Rockford, Ill.). After transfer to Immobilon P membranes (Millipore Co., Bedford, Mass.), immunoblotting was carried out with an antibody raised against PhoA and detected with an antiserum to rabbit immunoglobulin G conjugated to alkaline phosphatase as described previously (43).

RESULTS

Bactericidal effect of *S. pneumoniae* on *H. influenzae*. The hypothesis that pathogens inhabiting the same host environment might generate growth-inhibitory substances was examined. Initial experiments tested the effect of culture supernatant of *S. pneumoniae* P394 and *H. influenzae* Rd on the growth of the other species. Aliquots of culture supernatant filtrates from one organism were added to a lawn of the other organism which had been spread on solidified medium which supports the growth of only that species. A zone of completely inhibited growth was observed when supernatants from *S. pneumoniae* were added to lawns of *H. influenzae*, while the inverse showed no observable effect on growth (data not shown). This demonstrated that *S. pneumoniae* produced a substance that inhibited the growth of *H. influenzae*. Similar results were obtained using three nontypeable clinical isolates of *H. influenzae*, as well as the type b isolate, Eagan. Likewise, unrelated *S. pneumoniae* clinical isolates of types 2, 6A, and 6B were all capable of inhibiting the above-mentioned strains of *H. influenzae*, demonstrating that the observed effect was not strain specific.

To test whether this growth-inhibitory effect was also bactericidal, both species were grown to mid-log phase and cocultured in liquid medium. When 10^8 CFU of *H. influenzae* Rd per ml were cocultured with 5 x 10^7 CFU of *S. pneumoniae* P394 per ml, the viable count of *H. influenzae* decreased to below detectable levels (10^4 CFU/ml) within 3 h, whereas the viable count of *H. influenzae* cultured in the absence of *S. pneumoniae* under the same conditions increased to 10^9 CFU/ml (Fig. 1). In contrast, the viable count of *S. pneumoniae* increased to 10^8 CFU/ml, whether cultured with *H. influenzae* or in the absence of *H. influenzae*. These observations showed that the substance produced by *S. pneumoniae* was not only inhibitory but also bactericidal against *H. influenzae*.

In similar dose-response experiments, 10^7 CFU of *S. pneumoniae* per ml reduced the number of *H. influenzae* from 10^8 to 10^4 CFU/ml within 3 h (Fig. 2). *S. pneumoniae* at 10^6 CFU/ml reduced the equivalent number of *H. influenzae* approximately 10-fold within 3 h. The growth of the equivalent number of *H. influenzae* with 10^5 CFU of *S. pneumoniae* per ml was comparable to that of *H. influenzae* grown in the absence of *S. pneumoniae*.

The bactericidal effect of *S. pneumoniae* is due to hydrogen peroxide production. Supernatants from cultures of *S. pneumoniae* treated with proteinase K or heated to 65°C for

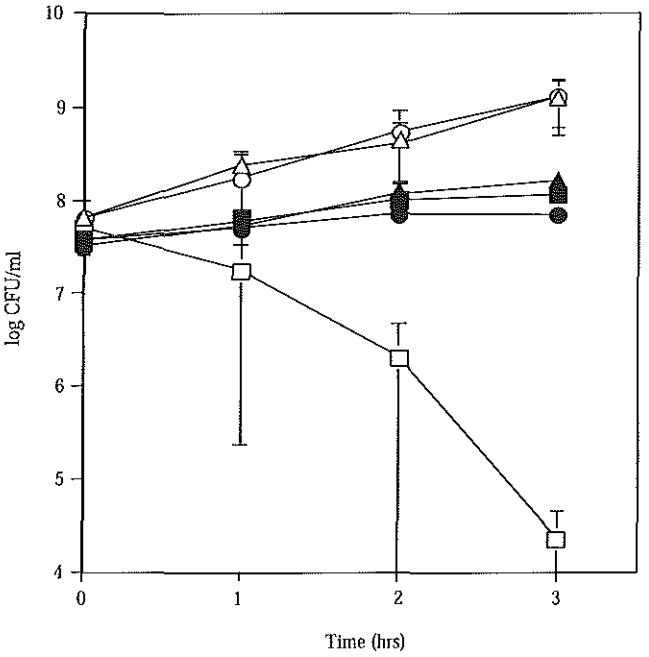

Fig. 1. Effect of coculture of *S. pneumoniae* P394 and *H. influenzae* Rd. Following growth to mid-log phase, *H. influenzae* was washed and incubated in sBHI containing heat-inactivated catalase either with (□) or without (○) *S. pneumoniae* for the time indicated, and viable counts were determined in duplicate on selective media. Viable counts of *S. pneumoniae* incubated in coculture with (■) or without (●) *H. influenzae* were determined in duplicate by plating on selective media. The same amount of active catalase (1,000 U/ml) was included during coculture of *S. pneumoniae* (▲) and *H. influenzae* (△). Values represent the average of three independent determinations in duplicate, and the error bars represent the standard deviations.

20 min retained inhibitory activity, suggesting that the inhibitory substance was not likely to be a protein (data not shown). In addition, the inhibitory effect was diminished when *S. pneumoniae* was grown under less-than-atmospheric levels of environmental oxygen (data not shown). It had previously been shown that *S. pneumoniae* makes substantial amounts of H_2O_2 when grown aerobically (2, 34). It was therefore suspected that the inhibitory effect of *S. pneumoniae* supernatant might be due to H_2O_2 production. Further support for this possibility came from the observation that the inhibitory effect was inversely proportional to the level of hemin in the growth medium (data not shown). Hemin had previously been shown to mitigate the effects of oxidative stress on *H. influenzae*, presumably because of its ability to decompose hydrogen peroxide (24, 27). Catalase, a heme-containing enzyme which specifically degrades H_2O_2, was then added to BHI plates at a concentration of 200 U/ml.

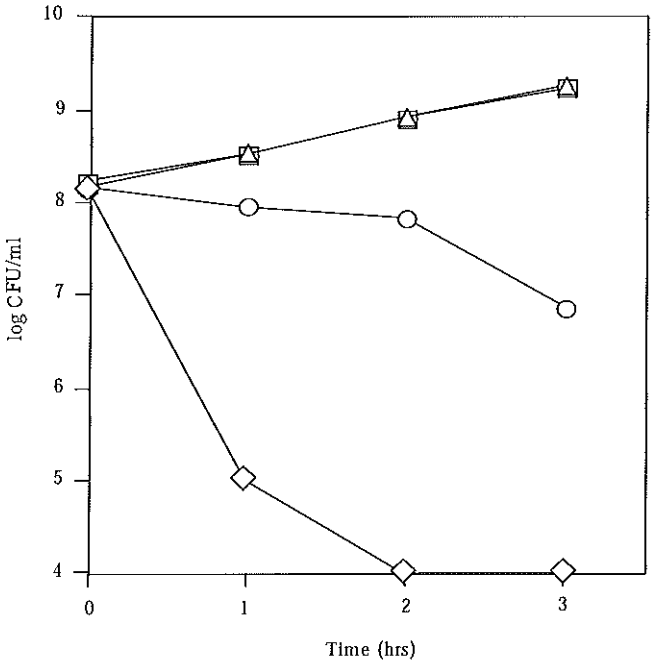

Fig. 2. Dose-dependent killing of *H. influenzae* Rd by *S. pneumoniae* P394. Following growth to mid-log phase, *H. influenzae* was washed and cultured alone (triangles) or with 10^5 (squares), 10^6 (circles), or 10^7 (diamonds) CFU of *S. pneumoniae* per ml and incubated in sBHI for the times indicated; viable counts were determined on selective media. Values represent the average of two independent determinations in duplicate

This eliminated the inhibitory effect of *S. pneumoniae* culture supernatants on *H. influenzae* (data not shown).

The effect of catalase on the bactericidal activity of *S. pneumoniae* was then explored using quantitative coculture experiments with bacteria grown in liquid medium. *H. influenzae* cultured with *S. pneumoniae* in the presence of 1,000 U of catalase per ml grew at the same rate as *H. influenzae* cultured alone, whereas heat-inactivated catalase (100°C for 20 min) was unable to eliminate the bactericidal activity of *S. pneumoniae* (Fig. 1). To confirm that hydrogen peroxide was responsible for the bactericidal activity of the pneumococcus, 10^8 CFU of *H. influenzae* per ml were cocultured with 5 x 10^7 CFU of an *S. pneumoniae* strain per ml in which the pyruvate oxidase gene (*spxB*) was insertionally inactivated. This mutant has previously been shown to produce <1% of the H_2O_2 of its parent strain, D39 (38). As expected, the *spxB* mutant was unable to kill *H. influenzae* in coculture experiments, in contrast to its parent strain D39 (Fig. 3). The growth of D39 and that of the *spxB* mutant were indistinguishable under these conditions.

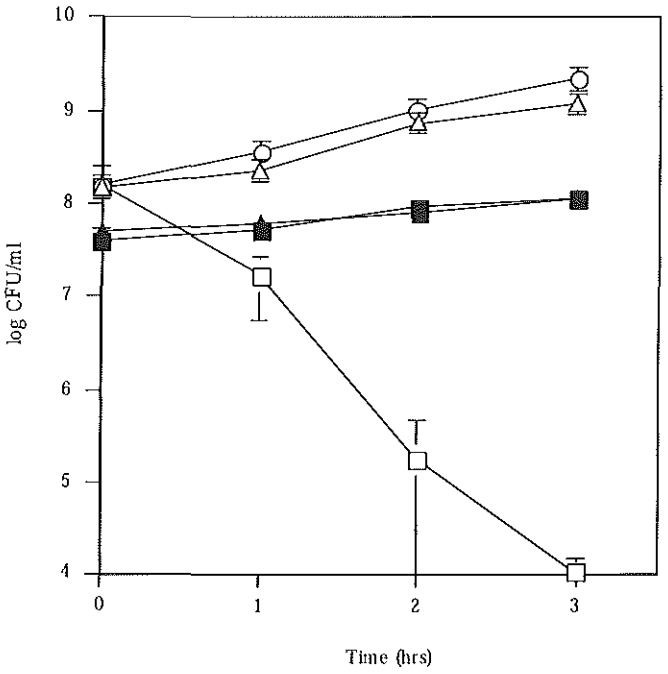

Fig. 3. Effect of coculture of *H. influenzae* Rd with *S. pneumoniae* D39 and its *spxB* mutant, P878. Following growth to mid-log phase, *H. influenzae* was washed and incubated in sBHI alone (○), with D39 (□), or with P878 (△) for the times indicated, and viable counts were determined in duplicate on selective media. Viable counts of D39 (■) or P878 (▲) incubated in coculture with *H. influenzae* were determined in duplicate by plating on selective media. Values represent the average of three independent determinations in duplicate, and the error bars represent the standard deviations.

Bactericidal effect of *S. pneumoniae* on other respiratory tract pathogens. The inhibitory effect of *S. pneumoniae* was tested on two other common inhabitants of the human respiratory tract: a clinical isolate of *Moraxella catarrhalis* and an unencapsulated mutant of a type b *N. meningitidis* strain (MC58C3). Catalase-reversible inhibition of *N. meningitidis* by supernatants from *S. pneumoniae* culture was observed on BHI agar. While an inhibitory effect of pneumococcal supernatant was not seen on lawns of *M. catarrhalis*, cross-streaking of *S. pneumoniae* and *M. catarrhalis* on BHI agar revealed a catalase-reversible inhibitory effect on *M. catarrhalis* only in the immediate vicinity of *S. pneumoniae*. Coculture experiments to examine the bactericidal effect on these species showed that 10^8 CFU of *N. meningitidis* per ml incubated with 5 x 10^7 CFU/ml *S. pneumoniae* for 1.5 h resulted in a catalase-reversible 45 ± 19% decrease in viable count compared to *N. meningitidis* cultured in the absence of *S. pneumoniae* (Fig. 4). *M. catarrhalis* grown at 10^8 CFU/ml in the presence of

5 x 10^7 CFU of *S. pneumoniae* per ml for 3 h resulted in a catalase-reversible 43 ± 21% decrease in viable counts compared to *M. catarrhalis* grown alone (Fig. 4). In contrast, the viable count of *S. pneumoniae* increased substantially when grown with either *N. meningitidis* or *M. catarrhalis* compared to *S. pneumoniae* grown alone (Fig. 4).

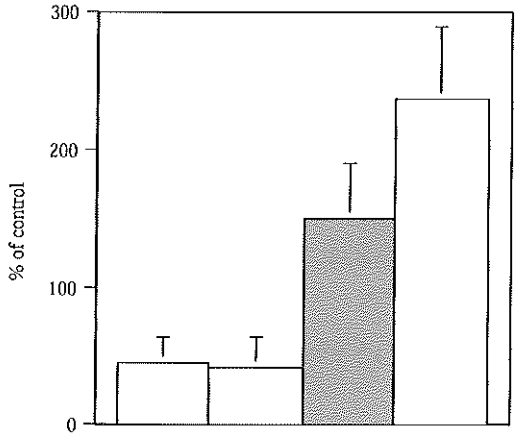

Fig. 4. Effect of coculture of *S. pneumoniae* P394 with either *M. catarrhalis* (Bc1) or *N. meningitidis* (MC58C3). Following growth to mid-log phase, *S. pneumoniae* (P394) was washed and incubated in BHI alone, with *N. meningitidis* (MC58C3) for 1.5 h, or with *M. catarrhalis* (Bc1) for 3 h. Viable counts of *N. meningitidis* (stippled bar) or *M. catarrhalis* (hatched bar) incubated in coculture with *S. pneumoniae* were determined in duplicate by plating on selective media. Viable counts of *S. pneumoniae* in coculture with *N. meningitidis* (black bar) or *M. catarrhalis* (white bar) were determined in duplicate on selective media. Values represent the change in viable count expressed as a percentage of a control culture containing that organism alone. Values are the average of three experiments, and error bars represent the standard deviations.

Hydrogen peroxide production and sensitivity to hydrogen peroxide. The relative sensitivities of *S. pneumoniae* P394 and the three other respiratory tract pathogens to hydrogen peroxide were examined using quantitative H_2O_2 challenge assays (Fig. 5). After a 30-min exposure to 0.1 mM H_2O_2, the survival of *S. pneumoniae*, *M. catarrhalis*, and *N. meningitidis* was unaffected, whereas the number of viable *H. influenzae* decreased by approximately twofold. At a concentration of 1.0 mM H_2O_2, the survival of *S. pneumoniae* and *M. catarrhalis* was unaffected, whereas the number of *H. influenzae* decreased approximately 2,000-fold, and the number of *N. meningitidis* decreased approximately 20-fold. At a concentration of 10 mM H_2O_2, *H. influenzae* and *N. meningitidis* decreased to undetectable levels (<100 CFU/ml), whereas the number of *S. pneumoniae* decreased only threefold, and *M. catarrhalis* was unaffected.

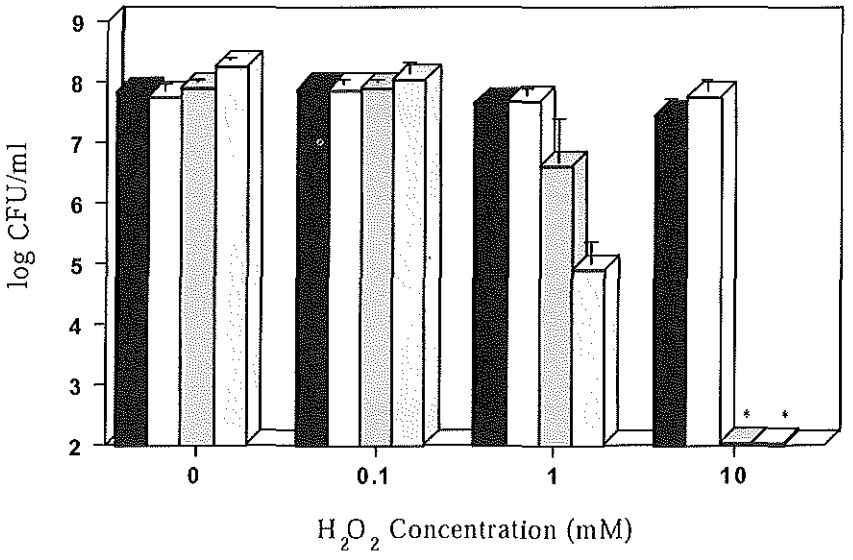

Fig. 5. Effect of H_2O_2 on the survival of *S. pneumoniae* (P394), *M. catarrhalis* (Bc1), *N. meningitidis* (MC58C3), and *H. influenzae* (Rd). Following growth to mid-log phase, *S. pneumoniae* (black bars), *M. catarrhalis* (white bars), *N. meningitidis* (stippled bars), or *H. influenzae* (hatched bars) were washed and incubated at 37°C in BHI or sBHI containing the indicated concentration of H_2O_2. After 30 min, viable counts were determined on BHI or sBHI plates containing 200 U of catalase per ml. Values represent the average of three independent determinations in duplicate, and the error bars represent the standard deviations. *, Below the limit of detection.

A survey of bacterial species was made to determine if the levels of hydrogen peroxide production and resistance exhibited by *S. pneumoniae* are unusual among human pathogens. Of the species tested for peroxide generation, only *S. pneumoniae* isolates exhibited production of detectable levels (>0.1 mM) of hydrogen peroxide using a horseradish peroxidase-phenol red assay (Table 1). Survival in different concentrations of exogenously added H_2O_2 varied widely among the species of gram-negative and gram-positive bacteria tested. The species most susceptible to growth inhibition and killing by H_2O_2 was *H. influenzae* (MIC, 0.4 mM; MBC, 0.5 mM). *N. meningitidis* was also relatively sensitive (MIC, 0.4 mM; MBC, 5.0 mM). *M. catarrhalis* was relatively insensitive to the effects of hydrogen peroxide (MIC, 1.1 mM; MBC, 160 mM). The pneumococcus was also relatively insensitive (MIC, 1.6 mM; MBC, 80 mM), thus explaining its ability to survive endogenously produced hydrogen peroxide.

Factors affecting hydrogen peroxide production by *S. pneumoniae*. Strains P62 and P64, two naturally occurring phase variants of the same strain, were tested for H_2O_2 production after it was determined by comparison of two-dimensional gel electrophoresis of whole bacterial proteins followed by micro-sequencing that the major difference in whole-cell protein expression was in the higher SpxB expression in P64 compared to P62 (Fig. 6, insert) (35a). Phase variation in SpxB expression correlated with difference in H_2O_2 generation, with P64 producing significant amounts of H_2O_2, whereas production by P62 was undetectable (Table 1). The bactericidal effect of these variants on *H. influenzae* was then compared in coculture experiments (Fig. 6). After 1.5 h of coculture, the decrease in the viable counts of *H. influenzae* in the presence of P64 was approximately 100-fold, whereas P62 had no effect. The production of H_2O_2 by the pneumococcus correlated with the concentration of O_2 in the environment, being decreased in microaerobic conditions (data not shown). In order to determine the effect of environmental oxygen on SpxB expression, Western blots were performed on lysates from strain P878, which contains an in-frame fusion of PhoA to SpxB, using an antibody to bacterial alkaline phosphatase. Equal amounts of whole-cell lysates of P878 cultured under various concentrations of O_2 and CO_2 were separated by SDS-PAGE, transferred to a membrane, and immunoblotted. A band corresponding to the SpxB-PhoA fusion protein was detected in samples grown aerobically but was almost completely absent from samples grown anaerobically (Fig. 7). The highest level of expression of SpxB was noted in the conditions of high oxygen and increased carbon dioxide, which correspond to the conditions expected of the mucosal surface of the respiratory tract.

DISCUSSION

This study documents the production of a soluble antimicrobial substance by *S. pneumoniae*. Several lines of evidence demonstrate that this substance is hydrogen peroxide. The effect of the pneumococcus in coculture experiments was completely eliminated by the addition of active but not inactivated catalase. A similar antimicrobial effect was reproduced by the addition of exogenous H_2O_2 at concentrations shown to be generated by the pneumococcus in liquid culture. This effect, furthermore, was absent in a pyruvate oxidase (*spxB*) mutant that synthesizes <1% of parental levels of H_2O_2 as well as a spontaneous variant that is downregulated in expression of SpxB (35a). Anaerobic growth conditions also lead to a diminished expression of SpxB which correlated with a loss of antimicrobial effect (data not

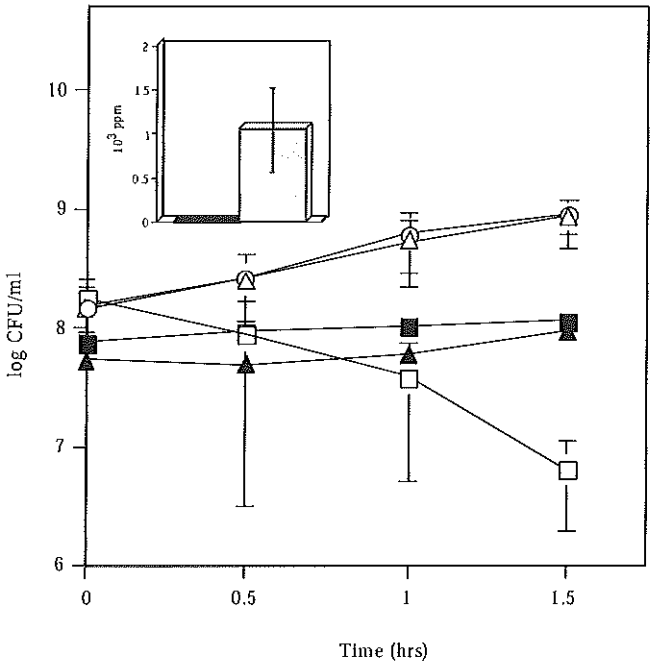

Fig. 6. Effect of coculture of *H. influenzae* Rd with *S. pneumoniae* opaque (P62) or transparent (P64) variants of a type 9V isolate. Following growth to mid-log phase, *H. influenzae* was washed and incubated in sBHI either alone (○), with P62 (△), or with P64 (□) for the times indicated, and viable counts were determined in duplicate on selective media. Viable counts of P62 (▲) or P64 (■) incubated in coculture with *H. influenzae* were determined in duplicate by plating on selective media. Values represent the average of three independent determinations in duplicate, and the error bars represent the standard deviations. (Inset) Relative expression of SpxB in *S. pneumoniae* variants P62 (black bar) and P64 (hatched bar) as determined by two-dimensional gel electrophoresis followed by mass spectrometric analysis. Results represent the average of four independent experiments, with error bars representing the standard deviations.

shown). Finally, the degree of antimicrobial effect against three species was proportional to their sensitivity to both growth inhibition and killing mediated by exogenous hydrogen peroxide.

Among the gram-positive ($n = 6$) and gram-negative ($n = 7$) species tested, the pneumococcus was the only species that generated concentrations of H_2O_2 that were >0.1 mM in liquid culture when at mid-log phase growth in aerobic conditions. For one of the *S. pneumoniae* strains tested, the average H_2O_2 concentration after 1 h of culture was 1.1 mM. This is consistent with the observation that *S. pneumoniae* produce approximately the same amount

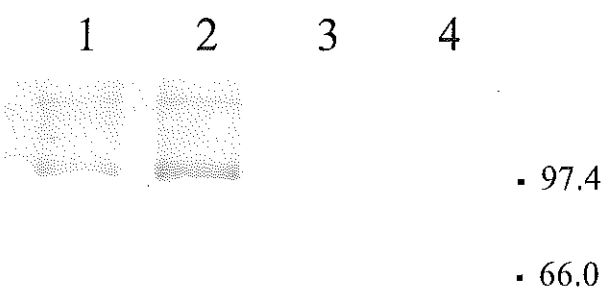

Fig. 7. Western blot showing the effect of environmental oxygen and carbon dioxide tension on pyruvate oxidase (SpxB) expression in *S. pneumoniae* P878, which contains an in-frame fusion to PhoA. Cell lysates of *spxB*::*phoA* mutant (P878) grown under 20% O_2–0.03% CO_2 (lane 1), 17% O_2–3% CO_2 (lane 2), or <0.01% O_2–10% CO_2 (lane 3) were electrophoresed on an SDS–10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and immuno-blotted with an antibody to PhoA. As a negative control, cell lysates from the parent strain (D39) grown under 17% O_2–3% CO_2 (lane 4) were included. Size markers are in kilodaltons.

of H_2O_2 per gram of total cellular protein as neutrophils during the oxidative burst (15). The calculated concentrations of H_2O_2 produced by *S. pneumoniae* in the present study agree with those previously reported for this species (2, 5, 38). Our results, furthermore, confirmed that the *spxB* mutant was deficient in H_2O_2 production (38). The mechanism that allows for the survival and growth of the pneumococcus, a catalase-negative organism, in substantial concentrations of hydrogen peroxide is unknown. *S. pneumoniae* contains NADH oxidase but lacks other systems involved in the oxidative stress response, such as OxyR (3). It was noted in this study that the mutant deficient in pyruvate oxidase activity often grew to a higher density in liquid culture. A similar effect on pneumococcal growth in liquid culture was observed in the presence of exogenous catalase and in coculture with *M. catarrhalis* or *N. meningitidis*, species that both produce high levels of catalase (37). Furthermore, the pneumococcus requires catalase for optimal growth on solid surfaces where the density of organisms is high (42). These observations support previous findings that endogenous production of hydrogen peroxide is permissive for growth but may have an adverse effect on its rate (2, 20, 34). This negative effect of hydrogen peroxide on growth raises the question as to why the pneumococcus, an organism that does not express catalase activity, synthesizes copious amounts of this highly toxic substance. It has been suggested that H_2O_2 generated by *S. pneumoniae* contributes to the pathogenesis of disease in the respiratory tract by its

cytotoxic effects on the epithelial barrier of the host (15, 19). This effect, however, required $\geq 10^8$ CFU/ ml, a density of bacteria unlikely to occur in the commensal state for this organism. In contrast, the antimicrobial effect was evident in coculture experiments with as few as 10^6 CFU/ml. Data presented here support the hypothesis that the pneumococcus generates unusually high amounts of hydrogen peroxide as a means of inhibiting and/or killing other species that may compete for the same environmental niche in the heavily colonized human nasopharynx.

Many lactic acid bacteria produce significant amounts of hydrogen peroxide during aerobic growth (50). In fact, several species of lactobacilli and oral streptococci have been shown to produce levels of H_2O_2 in liquid culture similar to that of *S. pneumoniae* (1 to 10 mM) (5, 16, 18, 47). Organisms shown to be killed or inhibited in vitro due to peroxide production by lactic acid bacteria include *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Corynebacterium diphtheriae*, and various other members of the oral flora (13, 16, 41, 50, 52). In the case of the pneumococcus, Colebrook was the first to describe its inhibitory activity by cross-streaking it with *N. meningitidis* and *M. catarrhalis* on solid medium (11). Similarly, McLeod and Gordon reported in 1922 the inhibition of growth of *S. aureus* due to *S. pneumoniae* culture supernatants, an effect they attributed to the presence of hydrogen peroxide (34). Our own study was able to take advantage of a genetically defined mutant that is essentially deficient in H_2O_2 production to confirm this hypothesis about the nature of the inhibitory substance generated by *S. pneumoniae* (38). Moreover, we demonstrate here that this antimicrobial effect may be a factor in the ability of the pneumococcus to compete against the other major pathogens residing in the upper respiratory tract of humans. The antimicrobial effect of the pneumococcus against three gram-negative, catalase-positive species that also colonize the mucosal surface of the human nasopharynx was assessed. The most dramatic effect was seen in coculture experiments with *H. influenzae*, where there was a 4-log decline in viable counts over 3 h due to the presence of 5×10^7 CFU of *S. pneumoniae* per ml. This was the most sensitive bacterial species among those tested to both the inhibitory and the bactericidal effects of the pneumococcus. If a similar effect occurs in vivo, this could at least in part account for the previously noted lower-than-expected rates of coinfection with *S. pneumoniae* and *H. influenzae* in otitis media and chronic bronchitis (25, 30). The inhibitory and bactericidal effects of H_2O_2 on *H. influenzae* occur despite the measurable expression of catalase by this species (8). In other words, a catalase-negative species, *S. pneumoniae*, is able to efficiently kill a catalase-positive species, *H. influenzae*, using H_2O_2. The level of

catalase activity as measured by the ability to catalyze the decomposition of hydrogen peroxide, however, varies widely from species to species, and *H. influenzae* seems to be an example of a catalase- positive organism with relatively low catalase activity as measured in vitro (7, 33). *H. influenzae* possesses only one gene for catalase, unlike the other gram-negative species *E. coli*, *Salmonella enterica* serovar Typhimurium, and *Shigella flexneri*, which produce two catalases (8). A previously reported catalase-deficient mutant of *H. influenzae*, strain AB2593 (Rd:: *hktE* ¯) was not significantly more sensitive to the antimicrobial effect of the pneumococcus compared to its parent strain, implying that catalase does little to protect *H. influenzae* under these conditions (data not shown) (8). *H. influenzae* may possess an impaired ability to upregulate catalase production in response to elevated levels of H_2O_2, possibly as a result of *H. influenzae*'s inability to synthesize protoporphyrin IX, the bio-synthetic precursor of heme, a required component of catalase (48). This finding is consistent with the observation that 10^8 CFU of exponentially growing *H. influenzae* produce only 5.7 U of catalase, and this expression level is induced only threefold by oxidative stress (8). Furthermore, the addition of *H. influenzae* to cultures of *S. pneumoniae* had only a small effect on the hydrogen peroxide concentration, suggesting that the endogenous production of catalase by *H. influenzae* was insufficient for these levels of H_2O_2 (data not shown). The effect of the pneumococcus was less dramatic against the meningococcus, where growth inhibition and minimal killing were observed after 1.5 h of coculture. When *M. catarrhalis*, a target species with markedly greater catalase activity, was tested, only a slight inhibitory bactericidal effect was evident after 3 h of coculture, although a catalase-reversible effect was noted with a higher density of pneumococci when the two organisms were cross-streaked on BHI agar.

In considering the contribution of hydrogen peroxide production to pneumococcal carriage, it should be noted that the studies presented here are based exclusively on in vitro effects. The synthesis of H_2O_2 by the pneumococcus in vivo has not been determined, although maximal expression of SpxB was noted in an oxygen and carbon dioxide rich environment, as would be expected on the surface of the upper respiratory tract. In addition, the antimicrobial effect correlated with variability in the expression of SpxB and was present in a variant with a transparent colony phenotype but not the opaque variant of the same isolate (35a). Only the transparent form has been shown to persistently colonize the nasopharynx in an animal model of carriage (42). This suggests that the increased production of H_2O_2 associated with this phenotype may contribute to its ability to efficiently colonize a host, whereas the opaque

phenotype may be outcompeted by the other flora. Another consideration in extrapolating these results to the situation in vivo is that host factors on the mucosal surface may act to inactivate bacterial hydrogen peroxide. In this regard, viridans streptococci, which may generate concentrations of hydrogen peroxide similar to that of *S. pneumoniae*, have been suggested to prevent colonization of gram-negative bacilli, including *H. influenzae*, in the human oropharynx by a mechanism that may be mediated in part by H_2O_2 production (39, 40). In addition, the *spxB* mutant of *S. pneumoniae* does not persist within the airway in an animal model of colonization in rabbits (38). Although the mechanism for this defect in carriage is unknown and there are several plausible explanations, it is possible that it results from an inability of the mutant to suppress local competitors. Future studies will address the significance of these observations to pneumococcal carriage and the maintenance of the normal microflora of the upper respiratory tract.

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Chapter 10

The putative proteinase maturation protein A of *Streptococcus pneumoniae* is a conserved surface protein with potential to elicit protective immune responses

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SUMMARY

Surface-exposed proteins often play an important role in the interaction between pathogenic bacteria and their host. We isolated a pool of hydrophobic, surface-associated proteins of *Streptococcus pneumoniae*. The opsonophagocytic activity of hyperimmune serum raised against this protein fraction was high and species specific. Moreover, the opsonophagocytic activity was independent of the capsular type and chromosomal genotype of the pneumococcus. Since the opsonophagocytic activity is presumed to correlate with in vivo protection, these data indicate that the protein fraction has the potential to elicit species-specific immune protection with cross-protection against various pneumococcal strains. Individual proteins in the extract were purified by two-dimensional gel electrophoresis. Antibodies raised against three distinct proteins contributed to the opsonophagocytic activity of the serum. The proteins were identified by mass spectrometry and N-terminal amino acid sequencing. Two proteins were the previously characterized pneumococcal surface protein A and oligopeptide-binding lipoprotein AmiA. The third protein was the recently identified putative proteinase maturation protein A (PpmA), which showed homology to members of the family of peptidyl-prolyl *cis/trans* isomerases. Immunoelectron microscopy demonstrated that PpmA was associated with the pneumococcal surface. In addition, PpmA was shown to elicit species-specific opsonophagocytic antibodies that were cross-reactive with various pneumococcal strains. This antibody cross-reactivity was in line with the limited sequence variation of *ppmA*. The importance of PpmA in pneumococcal pathogenesis was demonstrated in a mouse pneumonia model. Pneumococcal *ppmA*-deficient mutants showed reduced virulence. The properties of PpmA reported here indicate its potential for inclusion in multicomponent protein vaccines.

INTRODUCTION

Streptococcus pneumoniae is an important human pathogen which causes meningitis, otitis media, sepsis, and pneumonia. The precise molecular mechanisms by which the pneumococcus invades and damages host tissues are not fully understood. For many years, the polysaccharide capsule has been recognized as the major virulence factor and consequently was considered an important vaccine candidate (for review see references 5 and 34). The use of a 23-valent vaccine containing capsular polysaccharides from pneumococci commonly causing disease has had limited effect in reducing the morbidity and mortality

associated with this organism (1, 16, 19, 41). The current pneumococcal vaccine strategy focuses on the use of conjugates, in which a limited number of capsular polysaccharides are linked to a carrier protein. The proteins in the conjugate vaccines cause a switch in the immune response to polysaccharides from T-cell independent to T-cell dependent. This results in an increase in the antibody response and the generation of memory T lymphocytes. Conjugate vaccines are more immunogenic in young children than polysaccharide vaccines (15, 18). Although the results of early trials look promising, the long-term efficacy is uncertain since large-scale vaccination may over time lead to a shift in serotype distribution towards capsular types that are poorly immunogenic or not included in the vaccine. Such a shift may be enhanced by the horizontal exchange of capsular genes, as described previously (8, 22, 23).

Over the past few years, much attention has been focused on the role of pneumococcal proteins in pathogenesis and protection. Proteins that are involved in the pathogenesis of infections by *S. pneumoniae* are considered interesting components for future conjugate or multicomponent protein vaccines. The immunological response against such proteins should provide protection against colonization and infection with *S. pneumoniae* strains of all capsular polysaccharide types. Immunization with pneumolysin (36), pneumococcal surface protein A (PspA) (33, 45, 53), pneumococcal surface adhesin A (PsaA) (44), and neuraminidase (28) clearly confers protection in animal models.

The purpose of this study was to identify additional pneumococcal proteins with abilities to elicit protective immune responses. We isolated a pool of hydrophobic, potentially surface-associated proteins of *S. pneumoniae* that were able to elicit cross-reactive, species-specific antibodies with opsonophagocytic activity. At least three distinct proteins contributed to the in vitro opsonophagocytic activity. Two proteins were the previously characterized surface proteins PspA and oligopeptide-binding protein AmiA. The third protein was identified as the putative proteinase maturation protein A (PpmA) (35a). The potential of PpmA to elicit protective immune responses and its role in the pathogenesis of pneumococcal infection are discussed.

MATERIALS AND METHODS

Bacterial strains, growth conditions and growth medium. The pneumococcal strains used in this study are described in Table 1. Pneumococcal strain FT231 was used for protein purification. Bacteria were grown to logarithmic growth phase (optical density at 550 nm = 0.3)

Table 1. Bacterial strains used in this study.

Species and strain	Relevant characteristic (s)	Source[a] or reference
S. pneumoniae		
FT231	Serotype 19, clinical isolate	F. Tenover, CDC, Atlanta, Ga.
D39	Serotype 2, clinical isolate	7
CDC205	Serotype 3, clinical isolate	J. Butler, CDC, Atlanta, Ga.
EF3296	Serotype 4, clinical isolate	C. Svanborg, UvG[b], Sweden
ATCC6306	Serotype 6A, clinical isolate	ATCC[c]
911320	Serotype 6A, clinical isolate	Our laboratory
950357	Serotype 9V, clinical isolate	Our laboratory
S1003	Serotype 11, clinical isolate	Our laboratory
ATCC6314	Serotype 14, clinical isolate	ATCC[c]
950312	Serotype 14, clinical isolate	Our laboratory
S1001	Serotype 15, clinical isolate	Our laboratory
800129	Serotype 18C, clinical isolate	Our laboratory
19F G	Serotype 19F, clinical isolate	Our laboratory
ATCC6323	Serotype 23F, clinical isolate	ATCC[c]
950110	Serotype 23F, clinical isolate	Our laboratory
S3003	Serotype 38, clinical isolate	Our laboratory
19F K	Unencapsulated variant of 19F G	Our laboratory
Rx1	Unencapsulated variant of D39	42
P376	Serotype 6A, opaque variant of isolate P303	27
P765	Serotype 6B, transparent variant of isolate P314	26
P62	Serotype 9V, opaque variant of isolate P10	51
R189	*ermAM* containing strain	2
MS9	D39, *ppmA::ermAM*	This study
S. bovis		
961008	Clinical isolate	Our laboratory
E. faecalis		
ATCC29212	Clinical isolate	ATCC[c]

[a]CDC, Centers for Disease Control
[b]University of Göteborg
[c]American Type Culture Collection

in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% yeast extract (Difco Laboratories; THY broth) at 37°C.

Extraction of surface-associated hydrophobic proteins of *S. pneumoniae*. Bacterial cells were harvested by centrifugation (1,500 x g, 15 min, room temperature) and washed three times with an equal volume of phosphate-buffered saline (PBS, pH 7.5). After the final wash the bacteria were resuspended in 1/25 the volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA). The cells were disrupted by ultrasonic treatment (Branson sonifier 250, Branson Ultrasonics, Danburry, Conn.). The method for extraction with sulfobetaïne 14 (SB14) was adapted from that of Schouls and colleagues (40). In brief, cell walls, membranes and other particulate material were collected by centrifugation at 48,400 x g for 20 min. The water-soluble cytoplasmic proteins were removed by washing the bacterial lysates five times with PBS. Pellets were resuspended in 150 mM NaCl and centrifuged for 20 min at 48,400 x g. The pellets were

incubated for 2 h at room temperature with 0.25% *N*-tetradecyl-*N*,*N*-dimethylammonio-1-propanesulfonate (SB14, Serva, Heidelberg, Germany) in the presence of 150 mM NaCl-10 mM $MgCl_2$-10 mM Tris-HCl (pH 8.0) with constant stirring. The hydrophobic, membrane-associated proteins were recovered as described by Wessel and Flügge (52). Protein concentrations were determined by the method of Bradford (13).

Protein gel electrophoresis and staining. One-dimensional sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out in the Bio-Rad minigel system with 13% polyacrylamide gels. The samples were dissolved in sample buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 10 mM dithiothreitol [DDT], 10% glycerol, 0.01% bromophenol blue indicator [Merck, Darmstadt, Germany]), boiled for 5 min and subjected to electrophoresis (39). Two-dimensional SDS-polyacrylamide gel electrophoresis includes separation of proteins by isoelectric point and by molecular weight, respectively. Isoelectric focusing (pI 4 to 7) was performed with a Multiphor II electrophoresis unit and Immobiline DryStrips (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's recommendations, but as modified by Rabilloud and colleagues (37). The proteins were separated in the second dimension by gradient (12 to 20%) polyacrylamide gel electrophoresis. Polyacrylamide gels were stained with Coomassie brilliant blue (CBB) (39).

Hyperimmune rabbit antiserum. Hyperimmune antiserum was raised against the hydrophobic, surface-associated protein pool and distinct CBB-stained two-dimensional protein gel spots. Shortly after introduction into a one-dimensional SDS-polyacrylamide gel, the SB14-extracted protein pool of *S. pneumoniae* FT231 was stained with CBB and excised from the gel. The total protein fraction, as well as the individual protein spots cut from the two-dimensional polyacrylamide gel, were washed three times with 0.1 M sodium acetate, 96% ethanol, ground into a fine suspension in 0.5 ml of PBS, and subsequently mixed with 0.5 ml of Freund's incomplete adjuvant (Pierce, Rockford, Ill.). New Zealand White rabbits were injected subcutaneously in four or five places. The primary injection was followed by three booster injections at 4-week intervals.

Indirect immuno-cytometric assay. Pneumococci were grown to logarithmic phase in THY broth at 37°C and washed three times in ice-cold PBS. The bacterial pellet was dissolved in 5% rabbit serum in PBS (10^7 bacteria in a 20-μl final volume) and incubated for 15 min at 4°C with shaking. After being washed twice with ice-cold PBS, the bacteria were incubated for 15 min at

4°C with 20 µl (1:5 dilution) of fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories, West Grove, Pa.) with shaking. Finally, the bacteria were washed twice with ice-cold PBS and resuspended in 100 µl of ice-cold fresh paraformaldehyde (0.5%) in PBS. The samples were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

Phagocytosis assay. Analysis of the opsonophagocytic activity of the sera was performed as described by Alonso DeVelasco et al. (4) and adapted by Jansen et al. (24). In our assay, *S. pneumoniae* was grown to logarithmic phase in THY broth without heat-inactivated human pooled serum and the bacteria were not inactivated. Phagocytosis was defined as the uptake and binding of fluorescein isothiocyanate-labeled bacteria by human polymorphonuclear cells (PMNs) because of opsonization with antiserum. The opsonophagocytic activity is defined as the reciprocal of the serum concentration at which 25% (unless otherwise stated) of the human PMNs were fluorescent.

Western blot analysis. The proteins separated by one-dimensional (0.5 µg) and two-dimensional (2.5 µg) SDS-polyacrylamide gelelectrophoresis were transferred to Immobilon-P membranes (Millipore Corporation, Bedford, Mass.) as described by Sambrook et al. (39). The immunological detection of immobilized proteins was performed as described before (35a).

Amino acid sequence analysis. For N-terminal amino acid sequence analysis, the proteins were separated by two-dimensional SDS-polyacrylamide gel electrophoresis as described above with a few modifications. Recrystallized SDS (Serva) was used for preparing the electrophoresis buffers, and sodium thioglycolate (100 mM) was added to the cathodal buffer compartment. The proteins in the gel were blotted to a Problot membrane (Applied Biosystems, San Jose, Calif.) with the Multiphor II system (Pharmacia Biotech) according to the manufacturer's instructions except that 0.02% β-mercaptoethanol was added to the blotting buffer. The blots were stained with amido black (Merck). Amino acid sequence analysis was performed with a model 473A protein sequenator (Applied Biosystems) as recommended by the manufacturer.
For mass spectrometric analysis, the proteins of interest were purified from the gel, digested with trypsin, and analyzed by mass spectrometry as described (35a). With Peptide Search (29), the deduced (partial) amino acid sequences were analyzed for matching sequences in all possible translation products of the December 1998 version of the unfinished pneumococcal

genome released by the Institute for Genomic Research (TIGR; http://www.tigr.org/data/s_pneumoniae/) to identify the proteins. With the BLAST algorithm (6), putative pneumococcal proteins were analyzed for similarity to sequences deposited in the November 1999 version of the nonredundant protein database at the National Center for Biotechnology Information (Washington, D.C.).

Immunoelectron microscopy. Colonies of *S. pneumoniae* strain FT231 were fixed in a mixture of 4% formaldehyde, freshly prepared from paraformaldehyde, and 0.1% glutaraldehyde in sodium cacodylate buffer, pH 7.4. The bacteria were embedded in 1.5% low gelling temperature agarose (Sigma), and fixation was continued for a total of 24 h. Small pieces of the agarose-embedded bacteria were dehydrated in ethanol in a CS Auto apparatus (Reichart, Vienna, Austria) with a progressive lowering of temperature and embedded in Lowicryl HM20 resin (TAAB, Reading, United Kingdom) at -40°C. Ultrathin sectioning was performed with an Ultracut E microtome (Reichart). Sections were collected on gold grids and immunolabeled by incubation with anti-PpmA rabbit serum and anti-PspA rabbit serum, respectively, diluted 1:200 with buffer A (1% normal goat serum, 1% bovine serum albumine [Biocell], 0.1% Tween 20 [Sigma] in Tris-buffered saline, [pH 8.2]) for two h at room temperature. Control specimens were incubated with normal rabbit serum and in buffer A without primary antibody. Sections were washed in five 50-µl droplets of buffer A for 5 min each and incubated for 3 h at room temperature in goat anti-rabbit IgG antibody labeled with 5-nm-diameter colloidal gold (Biocell, Cardiff, United Kingdom) diluted 1:100 with buffer A. Final washing was done twice with buffer A and five times in distilled water. Sections were stained with uranyl acetate and examined with a Philips CM12 electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV.

Nucleotide sequence analysis of *ppmA*. Nucleotide sequencing was performed on *ppmA* from the pneumococcal strains D39, CDC205, EF3296, P376, P765, 950357, P62, S1003, 950312, S1001, 800129, FT231, 19F G, 950110, S3003, and Rx1. Two PCR products of 640 and 643 nucleotides, that cover the whole gene were generated with an overlap of 140 nucleotides. The primers 5'-TCTCATGCTTCGTAAAAATG-3' and 5'-AGCAAAATCAGCACCTTCTG-3' were used to amplify the 5' part of *ppmA*. The primers 5'-CTGAATTGACAGATGAAGCC-3' and 5'-CCTTGTACTATGCGTTTTATTG-3' were used to amplify the 3' part of *ppmA*. PCR amplification of *ppmA* was performed in a 100 µl PCR mixture as described for penicilin-

binding protein genotyping (35). The PCR products were purified by sodium acetate precipitation to remove the unincorporated nucleotides and primers (39). Purified templates were sequenced with the Thermo Sequenase dye terminator cycle sequencing premix kit (Pharmacia Amersham, Roosendaal, The Netherlands) and 50 pmol of each PCR primer. Sequencing was performed on the Applied Biosystems Prism 377 (PE Applied Biosystems, Nieuwerkerk, The Netherlands)

Cloning and insertional inactivation of *ppmA*. *ppmA* was amplified from *S. pneumoniae* strain D39 genomic DNA by PCR with primers pmpp-FW1 (5'-GTTTGGAATTCGCAAGCAAATCACTCTCC-3') positioned at nucleotides 369 to 340 upstream of the ATG start codon and pmpp-REV1 (5'-CAGTAGGATCCTTGTACTATGCGTTTTATTG-3') positioned at nucleotides 1073 to 1104 downstream of the ATG start codon. The forward and reverse primers contain *Eco*RI and *Bam*HI recognition sequences, respectively. The amplified *Eco*RI-*Bam*HI digested *ppmA* DNA fragment was cloned into pBluescript KS+ (pMS1) and transformed into *Escherichia. coli* DH5α (39). A *ppmA* mutant was constructed by insertion of an erythromycin resistance cassette (*ermAM*) in the gene. *ermAM* was amplified from *S. pneumoniae* strain R189 genomic DNA with primers ermAM-FW1 (5'-AAAGTTCGAAGCTTAAGTTCAAAACTACTTGCCC-3') and ermAM-REV1 (5'-AAAGCTGCAGTTCGAATGTCTTCTCACCTTTAG-3'). The amplified *ermAM* DNA fragment was *Csp*45I-digested and cloned into the *Csp*45I site of *ppmA* (nucleotide position 64 downstream of the ATG start codon) of pMS1 (pMS2). The *Eco*RI-*Bam*HI digested pMS2 DNA fragment was used to transform *S. pneumoniae* D39 as described previously (54). To confirm that *ppmA* was inactivated by *ermAM*, chromosomal DNA was analyzed by PCR with primers pmpp-FW1 and pmpp-REV1. Expression of PpmA was assessed by Western blot analysis with PpmA antibodies. The *ppmA* mutant MS9 was used in the described experiments.

Mouse pneumonia model. Preparation of the challenge dose and intranasal challenge of mice were performed as described before (25). Significant differences in the survival time of mice challenged with the *ppmA* knockout mutants and its parent strain D39 were determined by the nonparametric Mann-Whitney *U* test, with significance set at a value of P of ≤ 0.05.

RESULTS

Surface-associated protein fraction of *S. pneumoniae* is able to elicit opsonophagocytic activity. Approximately 30 polypeptides were isolated by the SB14 extraction procedure in relatively high concentrations, as shown by two-dimensional SDS-polyacrylamide gel electrophoresis (Fig. 1). Immunocytometric analysis demonstrated that serum raised against

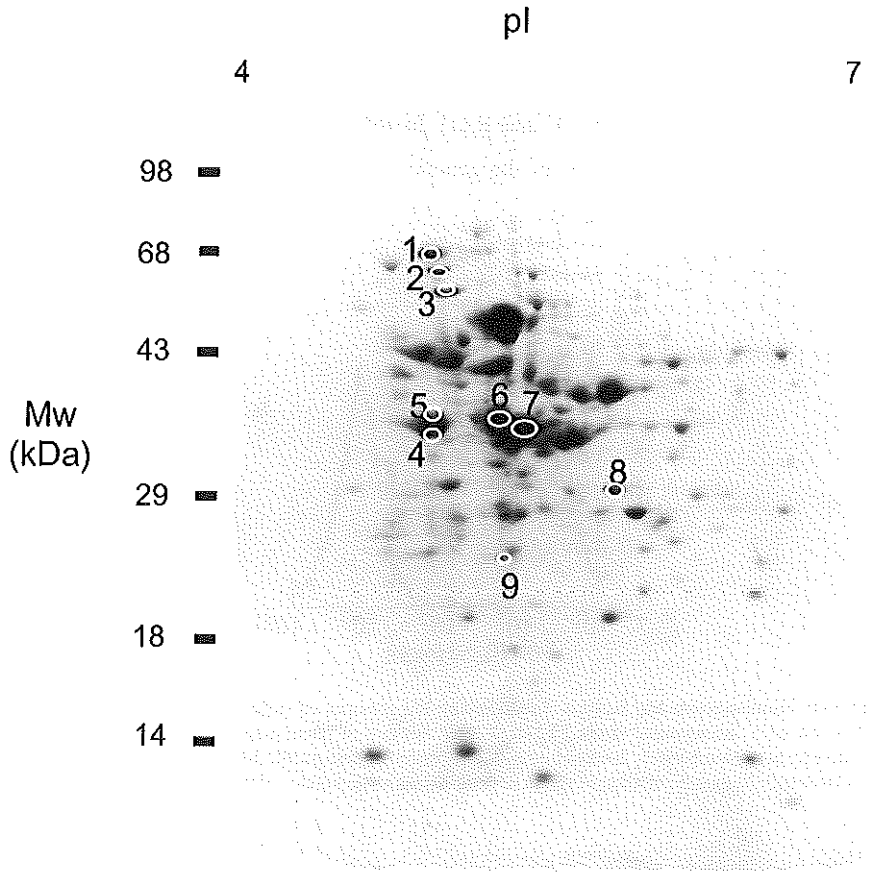

Fig. 1. Two-dimensional analysis of SB14-extracted surface-associated proteins from *S. pneumoniae* strain FT231. The proteins were separated by isoelectric focusing (pI 4 to 7) and gradient SDS-polyacrylamide gel electrophoresis (10 to 100 kDa) and stained with CBB. Circles mark the proteins recognized by serum raised against the SB14-extracted proteins. The numbers 1 to 9 refer to the proteins discussed in the text.

the extracted proteins recognized components at the surface of pneumococcal cells of the homologous strain FT231 (Fig. 2) and seven other pneumococcal strains (D39, EF3296, 911320, 950357, 800129, 19F, and 950110) that represent eight clinically important serotypes (types 2, 4, 6A, 9V, 14, 18C, 19F, and 23F, respectively) and display seven distinct genotypes (M. Sluijter, unpublished data). The in vitro serum opsonophagocytic activity was high ($50\%^{-1}$) when determined with the homologous pneumococcal strain FT231 (Fig. 3A). In addition, the serum was invariably opsonophagocytically active against six genotypically distinct pneumococcal strains (EF3296, ATCC6306, ATCC6314, FT231, 19F G1.1, and ATCC6323) (M. Sluijter, unpublished data) representing serotypes 4, 6A, 14, 19, and 23F and two unencapsulated strains (19F K1.1. and Rx1). In contrast, a low serum opsonophagocytic activity was found against two strains of the genetically closely related species *Streptococcus bovis* and *Enterococcus faecalis* (Fig. 4).

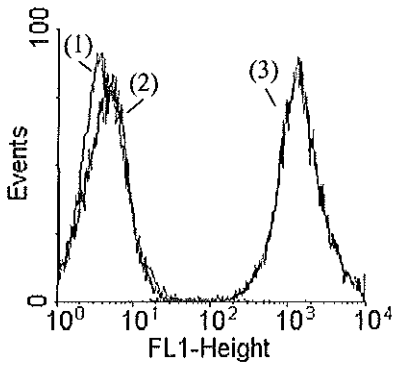

Fig. 2. Indirect immunocytometric analysis demonstrating the presence of surface-exposed epitopes in the SB14 protein fraction of *S. pneumoniae* strain FT231. *Y* axis, number of pneumococci analyzed. *X* axis, degree of immunofluorescence. Numbers: 1, bacterial auto-fluorescence; 2, nonspecific binding of fluorescein-conjugated goat anti-rabbit IgG; 3, specific binding of serum to components at the surface of pneumococci.

PspA, AmiA, and PpmA contribute to serum opsonophagocytic activity. Two-dimensional Western blot analysis was performed to identify the proteins that were serologically recognized at the surface of pneumococci and responsible for the in vitro opsonophagocytic activity. The hyperimmune rabbit serum recognized nine proteins, designated 1 to 9 (Fig. 1). Monospecific rabbit sera were raised against the individual proteins. The monospecific sera raised against proteins 2, 3, and 4 were able to facilitate phagocytosis of pneumococci with an opsonophagocytic activity of 0.5, 0.7 and $5\%^{-1}$, respectively (Fig. 3B to D). Protein 2 was analyzed by N-terminal amino acid sequencing, and proteins 3 and 4 were analyzed by mass

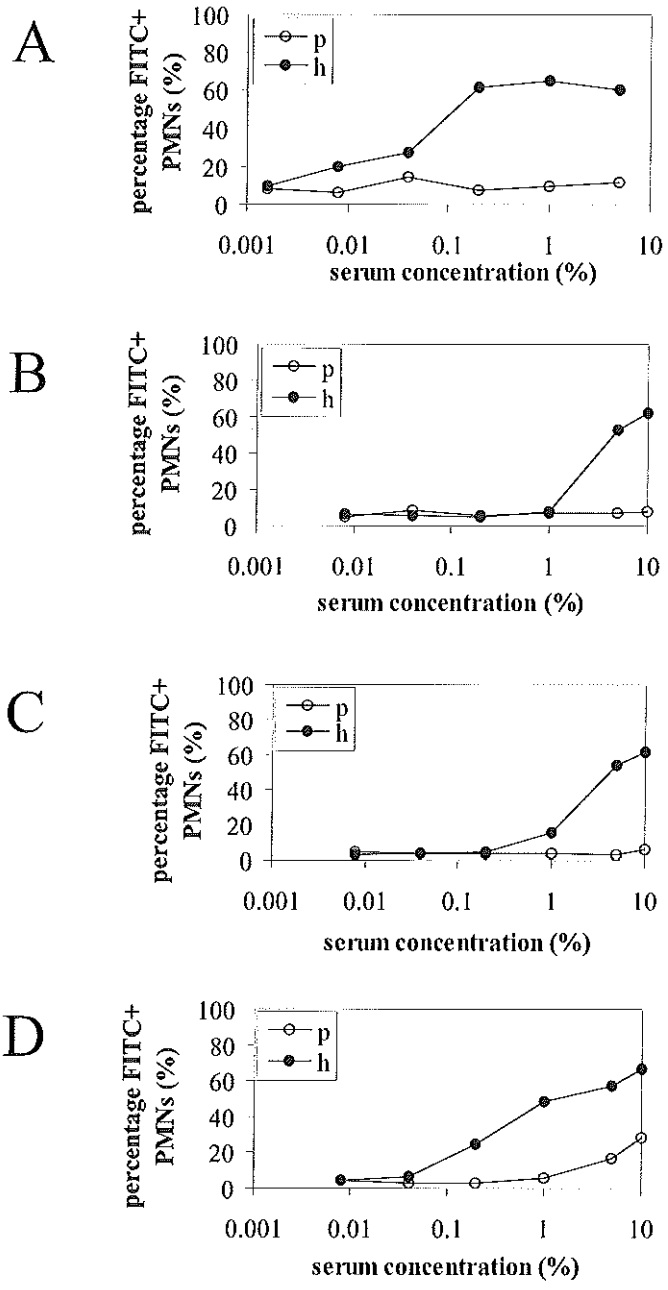

Fig. 3. The opsonophagocytic activity of hyperimmune rabbit sera raised against the surface-associated pneumococcal protein fraction (A) and sera raised against the proteins 2 (B), 3 (C), and 4 (D) using the homologous strain FT231. The percentage of fluorescent human PMNs was determined at various serum concentrations. p: pre-immune serum; h: hyperimmune serum.

spectrometry. Eighteen of the first 21 amino acids of protein 2 were successfully identified (Table 2). The amino acid sequence was identical to that of PspA as published by McDaniel and colleagues (GenBank accession number AAC62252) (31). The molecular size of PspA deduced from the two-dimensional protein gel was approximately 65 kDa and correlates with the size range of PspA (60 to 200 kD) (17, 50). Mass spectrometric analysis of protein 3 resulted in three peptides that were identical to AmiA (GenBank accession number P18791) (Table 2) (30). Mass spectrometric analysis of protein 4 resulted in 12 peptides (Table 2) that were part of a hypothetical translation product present in the TIGR pneumococcal genome encoding a protein of 322 amino acids (7,659 to 8,597, contig 33). The calculated size of this hypothetical protein (35.4 kDa) correlated with the size of protein 4 (approximately 35 kDa) deduced from the protein gel. This protein has been described before and designated putative proteinase maturation protein A (PpmA) due to its homology to proteinase maturation proteins (PrtM) of lactic acid bacteria (35a).

Table 2. Partial amino acid sequences of surface proteins 2, 3, and 4 identified by amino acid sequence analysis[a].

protein no.	name	Start-end position	Sequence
2	PspA	1-21	EEAPVAXQXKAEKDYDAAVXK
3	AmiA	40-49	1. VYTADPETLD
		148-159	2. DYLSGTSTDFST
		387-394	3. VAAQLPAY
4	PpmA	51-59	1. PSAQQVLLN
		64-66	2. QVF
		74-83	3. LDDQEVDDTL
		120-123	4. LAVQ
		125-137	5. VAEAELTDEAYQQ
		142-146	6. YTPDV
		161-165	7. EVLEQ
		195-198	8. FDSA
		221-235	9. VLTATGTQAYSSQYY
		248-251	10. NLDD
		260-264	11. LLTQQ
		268-277	12. STFVQSLLGK

[a]It is not possible to distinguish between Ile and Leu, Gln and Lys, and between Phe and oxidized Met by mass spectrometry.

PpmA is located at the surface of *S. pneumoniae*. Since the three proteins PspA, AmiA and PpmA are able to elicit opsonophagocytically active antibodies, they are presumed to be surface associated. The monospecificity of PpmA rabbit serum was confirmed by Western blot analysis. PpmA serum recognized a single protein band with the correct molecular size (35 kDa) in whole-cell lysates (Fig. 5). We performed indirect immunoelectron microscopy

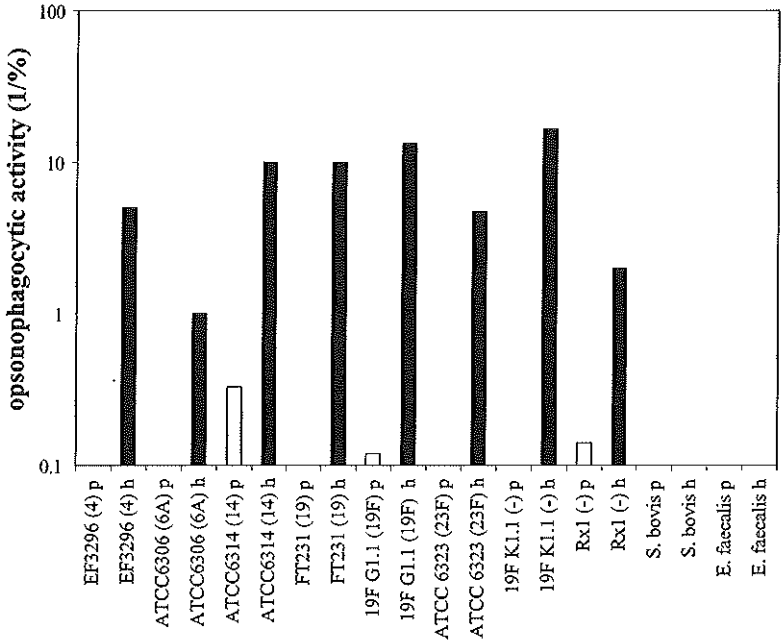

Fig. 4. Opsonophagocytic cross-reactivity of hyperimmune rabbit serum raised against the pneumococcal protein fraction using the heterologous pneumococcal strains EF3296, ATCC 6306, ATCC 6314, FT231, 19F G1.1, ATCC 6323, 19F K1.1, and Rx1, *S. bovis* strain 961008, and *E. faecalis* strain ATCC 29212. The serotype of the pneumococcal strains is indicated in parentheses. *Y* axis: opsonophagocytic activity, defined as the reciprocal of the serum concentration at which 25% of the human PMNs were fluorescent by phagocytosis of fluorescein isothiocyanate-labeled bacteria. p, pre-immune serum; h, hyperimmune serum raised against the surface-associated protein fraction.

to identify the subcellular location of PpmA. For this purpose, anti-PpmA serum was used. Rabbit serum raised against PspA and normal rabbit serum were used as positive and negative controls, respectively. Immunoelectron microscopy demonstrated that both PpmA and PspA antibodies bound to the surface of pneumococci (Fig. 6).

PpmA is able to elicit species-specific opsonophagocytic antibodies that are cross-reactive against various pneumococcal strains. The PpmA antibodies were shown to facilitate phagocytosis of eight genetically distinct pneumococcal strains representing serotypes 4, 6A, 9V, 14, 18C, 19F, and 23F. Preimmune rabbit serum was only opsonophagocytically active when the unencapsulated variant of strain 19F was used. The opsonophagocytic activity of the PpmA antibodies was very low when *S. bovis* and *E. faecalis* were used (Fig. 7).

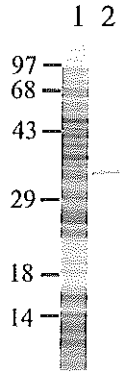

Fig. 5. Specificity of PpmA rabbit serum. Whole-cell lysates of *S. pneumoniae* strain FT231 were separated by one-dimensional protein gel electrophoresis, stained with CBB (lane 1) and analyzed by western blotting with PpmA serum (lane 2). The positions of the size markers are indicated in kilodaltons.

***ppmA* is conserved among pneumococcal strains.** DNA sequence analysis of *ppmA* from 16 pneumococcal strains representing 15 distinct genotypes and 13 serotypes, and including the seven serotypes that cause most of the infections in young children, revealed limited genetic variation. The variation in *ppmA* was randomly distributed, and most of the point mutations were synonymous. Compared to the TIGR genome sequence, we found variability in six nucleotides: A33G (n = 1), T81A (n = 10), T81G (n = 2), C87T (n = 1), T114C (n = 13), G146A (n = 15), T339C (n = 3) and G818A (n = 1). Except for G146A and G818A, none of the point mutations resulted in an amino acid substitution. Mutation G146A results in a Ser49Asp substitution, and mutation G818A results in a Ser272Asp substitution.

PpmA plays a role in the pathogenesis of pneumococcal infections in vivo. A *ppmA* knockout mutant of strain D39 was generated by insertion mutagenesis. Interruption of *ppmA* in erythromycin-resistant transformants was confirmed by PCR analysis (data not shown). In addition, absence of PpmA expression was demonstrated by Western blot analysis (data not shown). To confirm that the *ppmA* mutation did not affect the in vitro growth rate, both the mutant and the parent strain were grown overnight on blood agar, inoculated into THY broth, and incubated at 37°C for 8 h. During this period, there was no significant difference in growth rate between the *ppmA*-deficient strain and wild-type D39, as judged from the optical density of the culture (data not shown). To determine the effect of inactivation of *ppmA* on virulence, mice were challenged via the intranasal route with strain D39 and the *ppmA* mutant, respectively. Mice challenged with the *ppmA* mutant survived significantly longer

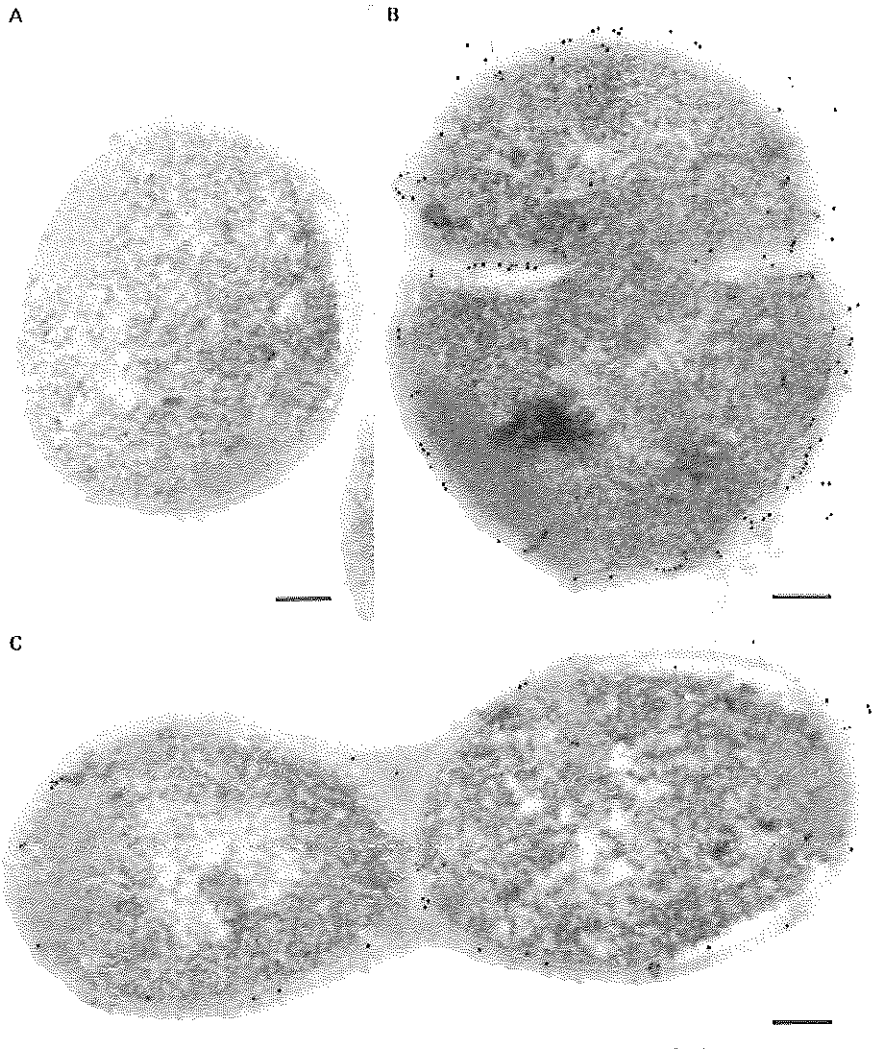

Fig. 6. Cellular localization of PpmA (A) and PspA (B) demonstrated by immunoelectron microscopy. Normal rabbit serum was used as negative control (C). Electron-dense immunogold particles are located mainly on the bacterial surface. Magnification x 1,125,000, scale bar, 100 nm.

than mice challenged with the parent strain D39 ($P = 0.023$); median survival times were 51.5 h and 33.0 h, respectively (Fig. 8). These data demonstrate that pneumococcal *ppmA* deficiency results in an extended survival time for mice during infection.

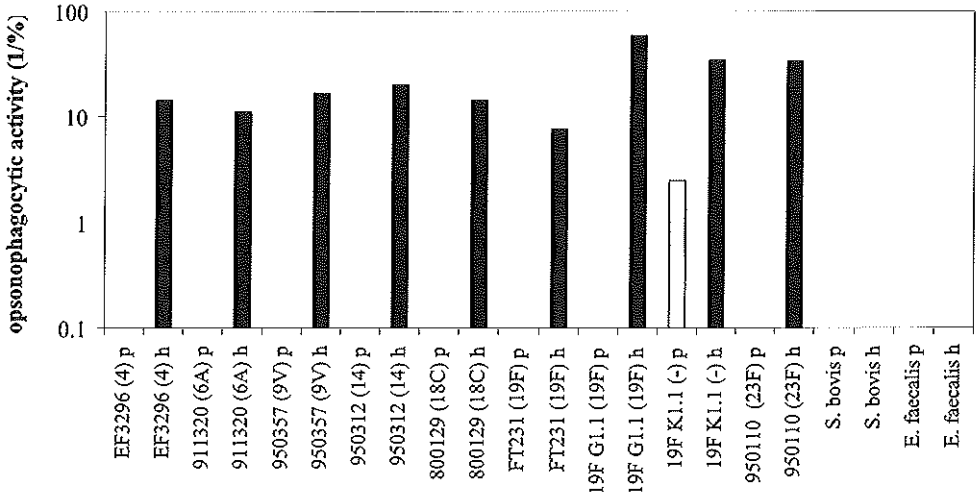

Fig. 7. Opsonophagocytic cross-reactivity of the monospecific hyperimmune sera raised against PpmA using the heterologous pneumococcal strains EF3296, 911320, 950312, FT231, 19F G1.1, 950110, 19F K1.1, and Rx1, *S. bovis* strain 961008 and *E. faecalis* strain ATCC 29212. The serotype of the pneumococcal strains is indicated in parenthesis. *Y* axis, opsonophagocytic activity, defined as the reciprocal of the serum concentration at which 10% of the human PMNs were positive by phagocytosis of fluorescein isothiocyanate-labeled bacteria. p, pre-immune serum; h, hyperimmune serum raised against PpmA.

DISCUSSION

Various pneumococcal proteins are displayed on the cell surface. These proteins have a wide range of functions, including adherence to host tissues, binding to specific immune system components, protein processing, nutrient acquisition, and uptake of DNA from the environment. Immunization with several pneumococcal surface proteins has been shown to confer protection against pneumococcal infection in animal models. These include PspA (45), PsaA (44), autolysin (9), and neuraminidase (28). To identify novel protection-eliciting pneumococcal proteins, we isolated a pool of SB14-soluble, potentially surface-associated proteins of *S. pneumoniae* strain FT231. This protein fraction was able to induce antibodies that facilitated phagocytosis in vitro. Phagocytosis is a major defense mechanism against pneumococci, and the induction of opsonophagocytic antibodies is presumed to correlate with

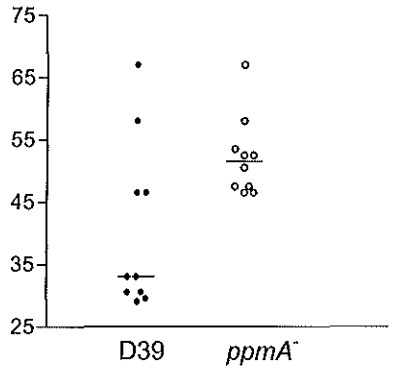

Fig. 8. Intranasal challenge of mice with *ppmA*-deficient *S. pneumoniae*. Groups of 10 mice were challenged with 10^6 CFU of *S. pneumoniae* strain D39 (solid circles) and its *ppmA*-deficient derivative (open circles). The survival time of each mouse is presented. Bars represent the median survival time for each group.

in vivo protection against *S. pneumoniae* infection (4). Among the proteins, at least three surface-associated proteins contributed to the in vitro opsonophagocytic activity.

The pneumococcal proteins were selected for hydrophobicity, immunogenic characteristics, and the ability to induce opsonophagocytic antibodies. One of the proteins was the previously characterized protein PspA. PspA inhibits complement activation and is proposed to exert a virulence function by recruitment of the alternative complement pathway, thereby reducing the effectiveness of complement receptor-mediated pathways of clearance (48). In addition, PspA functions as a lactoferrin-binding protein and is suggested to be involved in iron uptake and thus to contribute to pneumococcal growth under iron-limited conditions, i.e., in the human host (21). Surface exposure of PspA has been demonstrated previously (32, 46) and this characteristic was confirmed in this study by immunoelectron microscopy. The immunogenic nature of PspA observed in this study has also been demonstrated in previous studies (32). In line with the ability of PspA-specific antibodies to induce opsonophagocytic activity against strains expressing distinct capsular types, PspA has shown to possess immune protective potential (14, 32) with cross-protection (17, 47).

The second protein that induced opsonophagocytic antibodies was AmiA, which is a membrane-bound lipoprotein in *S. pneumoniae* (2) and part of the AmiA-AliAB oligopeptide permease that mediates the uptake of oligopeptides (2, 3). Since *S. pneumoniae* is auxotrophic for valine, leucine, arginine, asparagine, histidine, and glutamine, uptake of oligopeptides is important from a nutritional point of view (43). So far, no data are available

on the possible protective abilities of this protein. Our data derived from phagocytosis experiments with AmiA antibodies are the first indications that AmiA may be protective against pneumococcal infections. The contribution of the AmiA-AliAB oligopeptide permease system to pneumococcal virulence is currently under investigation.

The third surface-associated protein which possessed the ability to induce opsonophagocytic antibodies was PpmA. PpmA is a recently identified pneumococcal protein with significant sequence homology to the proteinase maturation protein (PrtM) of lactic acid bacteria (35a). Like PrtM from lactic acid bacteria (20), PpmA contains an N-terminal signal sequence, which serves as a label for translocation and cell membrane anchoring. In this study, the surface location of PpmA was confirmed by immunoelectron microscopy. The protein was able to induce antibodies in rabbits with opsonophagocytic activity. Although the affinity of the antibodies is unknown, the higher opsonophagocytic activity of PpmA antibodies compared to PspA and AmiA antibodies indicates the presence of relatively more PpmA molecules at the surface of the pneumococcus. Importantly, the opsonophagocytic activity of the PpmA antibodies was species specific and cross-reactive among heterologous pneumococcal strains. The observed opsonophagocytic activity of preimmune serum when the unencapsulated pneumococcal strain 19G K was used might be due to the absence of the capsule leading to the exposure of epitopes that are recognized by the preimmune serum. The cross-reactivity of the PpmA antibodies was in line with the limited sequence variation of *ppmA*. Like PrtM, PpmA is suggested to function as a membrane-bound isomerase (35a). PrtM is a *trans*-acting protein involved in the processing of precursors of serine protease PrtP into active enzymes (49) and belongs to the family of peptidyl-prolyl *cis/trans* isomerases. These enzymes are thought to assist in protein folding by catalyzing the *cis/trans* isomerization of the peptidyl-prolyl bonds in peptides and proteins (38). However, the pneumococcal protein(s) that is activated by PpmA is currently unknown. The differential expression of PpmA in phenotypic variants of *S. pneumoniae* indicates that PpmA may play a role in the pathogenesis of pneumococcal infections (35a). In the transparent phenotype that is selected for during nasopharyngeal colonization, PpmA is more prevalent, and therefore the protein may be involved in adherence through maturation of surface components or by the activation of proteases or other secreted proteins. In this study, PpmA was demonstrated to be involved in virulence. Inactivation of *ppmA* significantly reduced the virulence of strain D39 for mice as judged by survival time after intranasal challenge. However, the *ppmA* mutant was not completely avirulent. Like D39 mutants deficient in the production of pneumolysin (12), PspA (10), NanA (10), and LytA (11) that were also reduced in virulence, the *ppmA*

mutant was still capable of killing mice in our animal model. The proposed role of PpmA in speeding up the folding reactions (*cis/trans* isomerization of the peptidyl-prolyl bonds) is consistent with the significant but limited reduction in virulence of the mutant strain. The rate of maturation of target proteins is presumed to slow in the absence of PpmA. This will subsequently result in a reduction but not elimination of target proteins that are modified in their biologically active configuration. Another explanation for the limited reduction in virulence of PpmA-deficient mutants might be the presence of other as yet unknown *cis/trans* isomerases that partially substitute for the PpmA activity. We conclude that PpmA contributes to pneumococcal virulence. Based on the surface location of PpmA and its ability to elicit protective species specific antibodies, we also conclude that PpmA may be an interesting candidate for inclusion in future multicomponent protein vaccines.

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Chapter 11

General discussion

Streptococcus pneumoniae continues to be a common cause of serious and life-threatening infections such as pneumonia, bacteremia, and meningitis, and of noninvasive infections such as otitis media and sinusitis (3). Until the 1960s, pneumococci were considered to be uniformly susceptible to penicillin. However, in the last 30 years, increasing numbers of penicillin-resistant pneumococcal clinical isolates with increasing MIC values have been isolated all over the world (25). The emergence of high-level resistance to penicillin, particularly in combination with other resistance determinants, is a serious threat for current treatment strategies. Therefore, much attention is focused on disease prevention. Several issues concerning the epidemiology of pneumococcal disease are of importance in order to develop good treatment and prevention strategies. In this respect, five questions may be raised. How does the organism spread within the human population? Are particular subgroups of the pneumococcus responsible for different clinical manifestations of infection? Do certain subgroups dominate within the pneumococcal population? If yes, what are the virulence characteristics and resistance profiles of these subgroups? Will the pneumococcal population change after the introduction of vaccines?

Epidemiological markers provide a means of distinguishing different subgroups within species and facilitate the answer to these questions about the epidemiology of pneumococcal disease. Serotyping and the analysis of antibiotic resistance profiles remain key methods to characterize pneumococci. In addition, several other methods have been developed to assist the identification of the relatedness between strains. In our laboratory, restriction fragment end labeling (RFEL) analysis is routinely used to generate an international data library of pneumococcal DNA fingerprints. This technique provides a high degree of discriminatory power, and RFEL profiles have proved to be relatively stable over time and are very suitable for computerized comparison. DNA typing methods such as RFEL analysis provide greater discrimination and complementary information in epidemiological surveys. BOX PCR fingerprinting also provides genomic information that is suitable to identify the relatedness between strains. This technique is used in our studies to validate the genetic clustering obtained by RFEL analysis. DNA fingerprinting of the penicillin-binding protein (PBP) genes is used to investigate the genetic relatedness of the penicillin resistance genes.

Three molecular epidemiological studies were performed to investigate the epidemiological dynamics of penicillin-nonsusceptible pneumococcal isolates. The first study (Chapter 2) describes an international epidemiological investigation of penicillin-nonsusceptible isolates originating from 16 countries all around the world. In addition, the nature of the increase in

prevalence of penicillin-nonsusceptible pneumococci in Poland (Chapter 3) and Thailand (Chapter 4) was studied. In both countries, penicillin resistance has rapidly emerged. The percentage of penicillin-nonsusceptible isolates has increased from 0 to 3% in 1990 to 1993 (5, 35) to 14.3% in 1996 (62) and 14.4% in 1997 (61) in Poland, and from 6.7% in 1978 (58) to 10.6% in 1987 (36) and 37.2% in 1992 to 1994 (8, 49, 60) in Thailand. The penicillin-nonsusceptible strains were characterized by serotyping, drug susceptibility testing, RFEL analysis and PBP genotyping. In the Poland and Thailand studies, the genetic relatedness identified by RFEL analysis was confirmed by BOX PCR typing.

A predominant genetic type that was identical to the genetic type of the pandemic clone 23F (48) was observed in 10 countries. In addition, the genetic type that was displayed by the pandemic clone 9V (23) was observed in six countries. Serogroup 6 strains (57) were also frequently observed. However, the genetic homogeneity of these strains is limited. This finding is consistent with the observations of Harakeh et al. (30), who have demonstrated genetic heterogeneity within serotype 6B pneumococci isolated in the United States. Using less stringent genetic cut-off values, the serogroup 6 clone was observed in seven countries. These observations suggest that the introduction and subsequent pandemic spread of clone 6B has occurred earlier in the antibiotic era compared with the international clones 23F and 9V. Consequently, the contribution of clone 6B to the worldwide increasing prevalence of penicillin-resistant pneumococci is at present presumed to be overruled by the rapidly spreading clones 23F and 9V. The emergence of the pandemic clones 9V and 23F was also clearly demonstrated in Poland and Thailand. We conclude that these clones are responsible for the high prevalence of penicillin resistance in these countries. In Poland, the nation-wide emergence of two novel multidrug-resistant pneumococcal clones that are presumed to be of Polish origin has also contributed to the increase in the prevalence of penicillin-nonsusceptible pneumococci.

Several genetic clusters identified in these epidemiological studies consisted of two or more serotypes. This indicates frequent horizontal transfer of capsular genes.

PBP genotyping identified type 01-01-01 as the most predominant PBP genotype in Thailand and in 11 countries included in the international study. This PBP type was displayed by many distinct genetic types, suggesting that frequent horizontal exchange of PBP genes has resulted in the spread of this PBP genotype among various pneumococcal genetic types. Within genetic clusters of the Polish isolates several PBP genotypes were observed, suggesting that PBP genotypes have spread in the Polish population of penicillin-nonsusceptible pneumococci by horizontal gene transfer.

Comparison of the pneumococci with penicillin MIC values <1 μg/ml and those with MIC values ≥1 μg/ml clearly demonstrated that the degree of genetic clustering using both RFEL typing and PBP typing is higher among the penicillin-resistant isolates displaying MICs ≥1 μg/ml. In addition, the penicillin-resistant pneumococci with MIC values ≥1 μg/ml were more frequently multiple-resistant to the antibiotics penicillin, cotrimoxazole, doxycycline, and erythromycin. These observations suggest that the epidemic behavior of penicillin-resistant strains is enhanced with increasing levels of penicillin resistance. Moreover, we hypothesize that horizontal co-transfer of antibiotic resistance genes other than PBP genes occurs frequently among pneumococci with a high level of resistance to penicillin.

The molecular mechanism of penicillin resistance in The Netherlands was studied in detail in Chapter 5. Pneumococcal isolates express six PBPs: high molecular weight PBP1a, PBP1b, PBP2a, PBP2b, and PBP2x and low molecular weight PBP3. High molecular weight PBPs have transpeptidase activity. PBP1a, PBP2a, PBP2b and PBP2x are involved in penicillin resistance in *S. pneumoniae*. PBP2b and PBP2x are the primary targets for β-lactam antibiotics. Low affinity PBP1a increases the resistance in strains with low affinity PBP2x and/or PBP2b. Many resistant isolates are modified in these three PBPs only (26). The genes conferring resistance have a mosaic structure, in which parts of genes are replaced by genes from other species, presumably through horizontal gene transfer (14, 25, 27). The conserved amino acid motifs SXXK (with active site serine), SXN and K(H)T(S)G are important for the transpeptidase activity, and are believed to be located at the active site cavity. If one or more amino acids change in conserved motifs or adjacent amino acids, β-lactams are often unable to bind efficiently to PBP, leading to resistance. Pneumococci that have acquired a certain resistance level can spread, and will subsequently dominate environments where exposure to β-lactams occurs frequently. In addition, β-lactam-resistant pneumococci can provide donor DNA for transformation of susceptible strains resulting in the acquisition and spread of resistance among different pneumococcal genotypes (15) (Chapter 3).
Restriction fragment length polymorphism (RFLP) analysis was previously performed to characterize *pbp1a*, *pbp2b* and *pbp2x* from penicillin-nonsusceptible strains isolated in The Netherlands (32). In this study, we characterized specific gene fragments of *pbp1a* (42), *pbp2b* (21) and *pbp2x* (38) representing part of the PBP penicillin-binding domains of these clinical isolates. Classification based upon sequence analysis of these *pbp* fragments correlated in general well with the classification according to RFLP analysis, i.e. the deduced

genetic clustering of the pneumococcal strains was comparable between both methodologies. The identified mutations in the *pbp* sequences of the Dutch isolates invariably matched with the mutations described in *pbp* sequences of penicillin-nonsusceptible pneumococci isolated in other countries (6, 7, 19, 20, 37, 39, 43, 55, 56, 66). This observation demonstrates that the majority of the mutations and genetic rearrangements that contribute to the penicillin-nonsusceptible phenotype of pneumococci present in The Netherlands were already available in the current data libraries. In addition, this supports the hypothesis that multiple clones of penicillin-resistant pneumococci have been imported in The Netherlands. The *pbp* sequences identified in this study clearly demonstrate the acquisition of resistance by DNA transfer between non-pneumococcal species (*S. mitis, S. sanguis, S. oralis*) and pneumococci. Interestingly, novel combinations of mosaic structures were identified suggesting horizontal exchange of *pbp* gene fragments among penicillin-nonsusceptible pneumococci.

A nation-wide molecular epidemiological survey was performed on penicillin-susceptible isolates (Chapter 6). The epidemiological characteristics of all *S. pneumoniae* strains isolated from patients with meningitis in The Netherlands in 1994 were investigated. Only few studies have documented genotype analysis of penicillin-susceptible strains (32, 54). The genetic clustering was much lower among the penicillin-susceptible isolates than among penicillin-nonsusceptible isolates reported in the studies described in Chapter 2-4, and in other studies (9, 31, 32). In addition, comparison of the penicillin-susceptible isolates with penicillin-nonsusceptible strains present in the international data library and representing 16 countries revealed no overlap.

Horizontal transfer of capsular genes also occurs frequently among penicillin-susceptible strains, as at least a quarter of the genetic clusters consisted of two or more distinct serotypes. Horizontal transfer of capsular genes was described in penicillin-nonsusceptible pneumococcal populations (Chapter 2-4). In addition, various other investigators have reported horizontal transfer of capsular genes in penicillin-nonsusceptible isolates (9, 31, 32). At present, this is the first study describing horizontal transfer of capsular genes among penicillin-susceptible pneumococci.

PBP genotyping demonstrated limited variation in *pbp1a*, *pbp2b* and *pbp2x* of penicillin-susceptible strains. The PBP genotypes 02-02-03, 02-02-71 and 02-02-02 were found most frequently.

Furthermore, the genetic relatedness within serotypes was investigated (Chapter 6). Only few studies have documented genotype analysis of serotype specific strains (28, 40). The genetic types of serotype 6A and 19F strains displayed most heterogeneity. In contrast, the genetic types of serotype 7F, 9V and 3 strains were found genetically related. Interestingly and in agreement with our observations, Canadian penicillin-susceptible isolates of serotype 3 and 7F were also demonstrated genetically more related than isolates of other serotypes (40). Moreover, invasive penicillin-susceptible serotype 3 isolates from the United Kingdom tend to be mutually more closely related than isolates within other serotypes: the majority of the epidemiologically non-related serotype 3 strains grouped within two genotypes (28).
We focused on the molecular epidemiological characteristics of epidemiologically unrelated serotype 3 pneumococci and extended our serotype 3 collection with isolates form the United States, Thailand and Denmark. RFEL analysis demonstrated that serotype 3 strains were genetically related in spite of the absence of epidemiological relatedness. The vast majority of the strains represented two distinct genetic clades. Furthermore, both clades harbored isolates from three countries: the United States, Denmark, and The Netherlands. This indicates the international spread of two successful clones. We hypothesize that this serotype has evolved recently. The few serotype 3 isolates not belonging to the main clusters are presumably the result of horizontal transfer of capsular genes.

The current vaccine consists of the 23 most prevalent pneumococcal serotypes. The use of this 23-valent vaccine has only provided a minor contribution to the decrease in morbidity and mortality associated with *S. pneumoniae*. Importantly, this vaccine is unable to elicit adequate antibody responses in children less than two years of age who are an important risk group (18). The current pneumococcal vaccine strategies concentrate on the use of conjugate vaccines, in which capsular polysaccharides are linked to a highly immunogenic carrier protein thereby switching the immune response against polysaccharides from T-cell independent to T-cell dependent. Consequently, the antibody response towards the polysaccharides is increased and a memory response is provided. However, the number of different capsular polysaccharide types that can be included in the vaccine is restricted (50). In this respect, the frequency of horizontal transfer of capsular genes is of obvious importance once pneumococcal conjugate vaccines will be introduced in the population. Horizontal transfer of capsular genes may decrease the long-term efficacy of pneumococcal conjugate vaccination. Indeed, preliminary data of conjugate vaccination studies have shown that vaccination results in a shift of serotype distribution towards those pneumococcal

polysaccharide types that are not present in the vaccine (17, 45). Such a shift may be enhanced by the frequent horizontal transfer of capsular genes (Chapter 2-4, 6), and may lead to the acquisition of non-vaccine capsular types by (penicillin- and multidrug-resistant) pneumococcal strains.

Increased expression of capsular polysaccharide results in a lower degree of competence (51, 65), whereby the capsule acts as a barrier that prevents competence factor from reaching its cellular target (67). Pneumococcal opsonisation with immune-protective anti-polysaccharide antibodies raised by vaccination are hypothesized to increase the steric hindrance by the capsule, and affect the efficiency of horizontal gene transfer. The effect of opsonisation on pneumococcal competence is reported in Chapter 7.

In vitro DNA transfer experiments demonstrated, that pneumococcal opsonisation with capsular type-specific antibodies did not affect the uptake of marker DNA. These data indicate that horizontal transfer of capsule loci is not inhibited in the presence of opsonizing antibodies and may provide an escape mechanism for pneumococci in vaccinated individuals. Since recombination events have the potential to diminish the long-term effectiveness of current conjugate vaccination strategies, molecular epidemiological studies are needed to monitor pneumococcal colonization and infection of individuals who will be vaccinated with conjugate vaccines.

During the last few years, much attention is focused on the role of pneumococcal proteins in the pathogenesis of pneumococcal infection and the potential of these proteins to elicit protective immune responses. Proteins that are involved in the pathogenesis of infections by *S. pneumoniae* are considered interesting components for future conjugate or multicomponent protein vaccines. The immunological responses against such proteins should ideally provide protection against colonization and infection with pneumococcal strains of all capsular polysaccharide types.

In Chapter 8, proteins were identified that are presumed to play a role in the infectious process. The pneumococcus undergoes spontaneous phase variation resulting in opaque and transparent colony forms. Differences in colony opacity have been shown to correlate with differences in virulence: the transparent variants are more capable of colonizing the nasopharynx, whereas the opaque variants show increased virulence during systemic infections. Phenotypic variation is an important mechanism allowing bacterial pathogens to adapt to different host environments. The ability to survive in the different host

environments is the result of molecular adaptation, e.g. changes in the expression of specific cell surface components (29, 64).

Protein expression patterns of *S. pneumoniae* phenotypic variants were compared. Three proteins were identified and characterized that are differentially expressed in the phenotypic variants. One of the proteins was overexpressed in the opaque phenotype. This protein revealed significant homology with elongation factor Ts (EF-Ts) of *Helicobacter pylori*. EF-Ts is essential for the elongation of the polypeptide chain during protein synthesis. The protein mediates the regeneration of EF-Tu•GDP into the active form EF-Tu•GTP. This active form of EF-Tu facilitates the entry of aminoacyl-tRNA to the ribosome enabling protein synthesis. Differential expression of EF-Ts has previously been described in the gram-negative bacterium *Coxiella burnetii* (53), the causative agent of Q fever. This obligate intracellular parasite replicates in distinct morphological forms that may allow potential life cycle variants to survive the harsh environment of the phagolysosome. Two distinct morphological forms of *C. burnetii* have been described, a large cell variant and small cell variant. Large cell variants have demonstrated to be metabolically more active than small cell variants (53). This is supported by data demonstrating that EF-Ts and EF-Tu were more prevalent in large cell variants (53). We hypothesize that the increased presence of EF-Ts in the opaque variants of *S. pneumoniae* indicates that, like in *C. burnetii*, the opaque variants are metabolically more active, which may explain the rapid invasive growth characteristics of these variants.

Two proteins were overexpressed in the transparent variant. One of these proteins was identified as pyruvate oxidase SpxB. Pyruvate oxidases are crucial for aerobic carbohydrate metabolism of several streptococci (16). This enzyme decarboxylates pyruvate to acetyl phosphate resulting in the release of H_2O_2 and CO_2. In *S. pneumoniae,* the release of H_2O_2 is mainly the result of SpxB activity since a *spxB*-defective mutant produces virtually no H_2O_2 in comparison with the parent strain (59). Therefore, the higher expression of SpxB in the transparent phenotype most likely explains the determined increased production of hydrogen peroxide. Spellerberg and colleagues have identified pyruvate oxidase as an indirect determinant of virulence in *S. pneumoniae* (59). A *spxB*-mutant has a decreased ability to colonize the nasopharynx in a rabbit model. This is reflected by the lower binding capacity to the nasopharyngeal cells, and to their glycoconjugate receptors. The decreased ability of *spxB*-defective pneumococcal mutants to colonize the nasopharynx, and the increased expression of SpxB in transparent variants suggests the potentially important role of SpxB for transparent variants to efficiently colonize the nasopharynx (59). The expression level of

SpxB is unlikely to directly determine colony morphology, since the *spxB*-mutant of D39 still varies in colony morphology (J. N. Weiser, unpublished data). Interestingly, pyruvate oxidase was identified to play a direct role in the bactericidal effect of the pneumococcus on *Haemophilus influenzae* and in the inhibitory effect on the growth of *Moraxella catarrhalis* and *Neisseria meningitidis* (Chapter 9). Expression of SpxB and, consequently, production of hydrogen peroxide was demonstrated responsible for these effects. The production of hydrogen peroxide is permissive for growth of the pneumococcus but may have an adverse effect on its rate (4, 34, 47). The mechanism that allows for the survival and growth of the pneumococcus, a catalase-negative organism, in substantial concentrations of hydrogen peroxide is unknown. *S. pneumoniae* produce approximately the same amount of H_2O_2 per gram of total cellular protein as neutrophils during the oxidative burst (22). The pneumococcus thus generates unusually high amounts of hydrogen peroxide and, thereby, inhibits and/or kills other species that may compete for the same environmental niche in the heavily colonized human nasopharynx. *H. influenzae* was the most sensitive bacterial species among those tested to both the inhibitory and bactericidal effects of the pneumococcus. If a similar effect occurs in vivo, this could at least in part account for the previously noted lower than expected rates of co-infection with *S. pneumoniae* and *H. influenzae* in otitis media and chronic bronchitis (41, 44). The increased production of H_2O_2 associated with the transparent phenotype may, therefore, contributes to its ability to efficiently colonize a host; as a result of the ability to suppress local competitors.

The second protein overexpressed in transparent variants revealed significant homology with the proteinase maturation protein PrtM of *Lactocobacillus paracasei*, a member of the family of peptidyl-prolyl *cis/trans* isomerases. *L. paracasei* and other lactic acid bacteria are used in the food industry for the production of a variety of fermented milk products. During growth, these bacteria produce cell envelope-located serine proteases that degrade caseins, the major proteins in milk. PrtM is a *trans*-acting protein involved in the maturation (processing into active proteins) of serine protease PrtP (63). PrtM belongs to the family of peptidyl-prolyl *cis/trans* isomerases that is thought to assist in protein folding by catalyzing *cis/trans* isomerization of peptidyl-prolyl bonds in peptides and proteins (52). The pneumococcal putative proteinase maturation protein A, PpmA, contains an N-terminal lipoprotein signal sequence, which suggests that PpmA, like PrtM (24), is membrane-bound. We hypothesize that PpmA also functions as a membrane-bound molecular chaperone. In *L. paracasei,* the gene encoding the serine protease PrtP is located immediately downstream of *prtM* (33). Both *prtM* and *prtP* were found to be transcribed from the same promoter region but in

opposite directions (63). In *S. pneumoniae,* no open reading frame homologous to a serine protease could be located in the direct vicinity of *ppmA*. Which pneumococcal proteins are activated by PpmA is currently unknown. The higher prevalence of PpmA in the transparent phenotype, which is selected for during nasopharyngeal colonization, suggests that PpmA is either directly involved in adherence through maturation of surface proteins with adherence properties or indirectly by the activation of proteases or other secreted proteins. The consensus lipoprotein signal sequence suggests that PpmA is located at the surface of the pneumococcus, and may play a role in the maturation of surface- or secreted proteins. Interestingly, this protein was also identified in a study aiming to identify pneumococcal proteins with potential to elicit protective immune responses (Chapter 10). In this study, a pool of SB14-soluble, potentially surface-associated proteins of *S. pneumoniae* strain FT231 was isolated. This protein fraction was able to induce antibodies that facilitated phagocytosis in vitro. Phagocytosis is a major defense mechanism against pneumococci, and the induction of opsonophagocytic antibodies is presumed to correlate with in vivo protection against *S. pneumoniae* infection (2). Among the purified proteins, at least three surface-associated proteins contributed to the in vitro opsonophagocytic activity. Two proteins were the previously characterized pneumococcal surface protein A (46) and oligopeptide-binding lipoprotein AmiA (1). PspA has previously shown to possess immune protective potential (13, 46). So far, no data are available on the possible protective abilities of AmiA. Our data derived from phagocytosis experiments with AmiA-antibodies are the first indications that AmiA may be protective against pneumococcal infection. The third protein revealed to be PpmA. The opsonophagocytic activity of the PpmA antibodies was species-specific and cross-reactive among heterologous pneumococcal strains irrespective of the capsular type. The cross-reactivity of the PpmA antibodies was in line with the limited sequence variation of *ppmA*. The study also included immunoelectron microscopy, confirming that PpmA was indeed associated with the pneumococcal surface as suggested in Chapter 8. The importance of PpmA in pneumococcal pathogenesis was demonstrated in a mouse pneumonia model. Inactivation of *ppmA* significantly reduced the virulence of strain D39 for mice as judged by survival time after intranasal challenge. However, the *ppmA* mutant was not completely avirulent. Like D39 mutants deficient in the production of other virulence factors (pneumolysin (12), PspA (10), NanA (10), and LytA (11)) that were also reduced in virulence, the *ppmA* mutant was still capable of killing mice in our animal model. The proposed role of PpmA in speeding up the folding reactions (*cis/trans* isomerization of the peptidyl-prolyl bonds) is consistent with the significant but limited reduction in virulence of

the mutant strain. The rate of maturation of target proteins is presumed to slow in the absence of PpmA. This will subsequently result in a reduction but not elimination of target proteins that are modified in their biologically active configuration. Another explanation for the limited reduction in virulence of PpmA-deficient mutants may be the presence of other as yet unknown *cis/trans* isomerases that partially substitute for the PpmA activity. Due to its potential to elicit protective immune responses irrespective of the capsular phenotypes, PpmA may be an interesting candidate for inclusion in future multicomponent protein vaccines.

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SUMMARY

Streptococcus pneumoniae (the pneumococcus) is a human pathogen that is a leading cause of invasive diseases including pneumonia, bacteremia and meningitis. The pneumococcus is also a major worldwide cause of otitis media and sinusitis. The incidence of pneumococcal disease is highest in young children, elderly and immunocompromised individuals. Until the 1960s, pneumococci were considered uniformly susceptible to penicillin. However, in the last 30 years, increasing numbers of penicillin-resistant pneumococcal clinical isolates have been isolated from all over the world. The emergence of high-level resistance to penicillin, particularly in combination with resistance to other antibiotics, is a serious threat for current treatment strategies. Therefore, much attention is focused on disease prevention. Several issues concerning the epidemiology of pneumococcal disease are of importance in order to develop good strategies for treatment and prevention. In this respect, four questions may be raised. How does the bacterium spread within the human population? Do certain subgroups dominate within the pneumococcal population? If yes, what are the virulence characteristics and resistance profiles of these subgroups? Will the pneumococcal population change after the introduction of vaccines? The current vaccine consists of the 23 most prevalent pneumococcal capsular polysaccharide types. To date, 90 pneumococcal capsular polysaccharides have been identified. The use of this 23-valent vaccine has only provided a minor contribution to the decrease in morbidity and mortality associated with *S. pneumoniae* infections. Importantly, polysaccharide vaccines are unable to elicit adequate antibody responses in children less than two years of age. The current pneumococcal vaccine strategies concentrate on the use of conjugate vaccines, in which capsular polysaccharides are linked to a highly immunogenic carrier protein thereby switching the immune response against polysaccharides from T-cell independent to T-cell dependent. This will result in an increased antibody response against the polysaccharides and will induce a memory response. However, the number of different capsular polysaccharide types that can be included in the conjugate vaccine is restricted. During the last few years, much attention is focused on the role of pneumococcal proteins in the pathogenesis of pneumococcal infection and the potential of these proteins to elicit protective immune responses. Proteins that are involved in the pathogenesis of infections by *S. pneumoniae* are considered interesting components for future conjugate or multicomponent protein vaccines.

The aims of my research project were both molecular epidemiological aspects of *S. pneumoniae*, and the identification of pneumococcal proteins involved in the pathogenesis of infections.

Three molecular epidemiological studies were performed to investigate the epidemiological dynamics of penicillin-nonsusceptible pneumococcal isolates. **Chapter 2** describes an international epidemiological investigation of penicillin-nonsusceptible isolates originating from 16 countries all around the world. The two most predominant genetic types correspond with a Spanish pandemic clone 23F and a Spanish-French international clone 9V. The pandemic behavior of these two clones was clearly demonstrated, since they were found in 10 and six different countries, respectively. Several genetic clusters consisted of two or more capsular types. This indicates frequent horizontal transfer of capsular genes. In addition, identical penicillin-binding protein (PBP) genoypes were often observed within distinct genetic types. This observation demonstrates a high frequency of horizontal DNA transfer of penicillin resistance genes. The vast majority of the strains belonging to the pandemic clones 23F and 9V shared one PBP type. We hypothesize that the clones 23F and 9V are responsible for the worldwide increase of penicillin-resistance, and may serve as an important genetic reservoir for susceptible pneumococci to acquire penicillin resistance.

A study on the epidemiologic nature of the increase in prevalence of penicillin-nonsusceptible pneumococci in Poland is described in **Chapter 3**. Four genetically distinct clusters of strains were identified among penicillin-nonsusceptible strains isolated in 1995 and 1996 in 16 towns across the country. Two clusters represented the pandemic clones 23F and 9V, which have recently emerged all over the world. The two other genetic clusters, which represented capsular types 23F and 6B, clearly predominated in the collection of Polish penicillin-nonsusceptible pneumococcal strains. Their Polish clonal origin is most likely, since the latter clusters did not match any of the genetic types of penicillin-nonsusceptible pneumococci collected in 15 other countries.

In **Chapter 4**, the epidemiologic nature of the dramatic increase in prevalence of penicillin-nonsusceptible pneumococci in Thailand was investigated. The most predominant multidrug-resistant pneumococcal clones were identical to the pandemic clones 23F and 9V. We conclude that these pandemic clones are primarily responsible for the increase in the prevalence of pneumococcal penicillin resistance in Thailand.

The molecular mechanism of penicillin resistance in The Netherlands was studied in detail in **Chapter 5**. Pneumococcal isolates express six PBPs. Many penicillin-nonsusceptible isolates are modified in PBP1a, PBP2b and PBP2x only. In this study, we characterized fragments of

the DNA sequence of *pbp1a*, *pbp2b* and *pbp2x* of Dutch clinical isolates. The pneumococcal strains were selected on the basis of differences in restriction fragment length polymorphism (RFLP) patterns of the complete genes *pbp1a*, *pbp2b* and *pbp2x*. Classification based upon sequence analysis of these *pbp* fragments correlated well with the classification according to RFLP analysis. The mutations in the *pbp* sequences of the Dutch isolates invariably matched with the mutations described in *pbp* sequences of penicillin-nonsusceptible pneumococci isolated in other countries. This observation supports the hypothesis that multiple clones of penicillin-resistant pneumococci have been imported and have spread in The Netherlands. Interestingly, novel combinations of mosaic structures were also identified indicating horizontal exchange of *pbp* gene fragments among penicillin-nonsusceptible pneumococci.

The molecular epidemiologic characteristics of penicillin-susceptible Dutch strains of *S. pneumoniae* were investigated in **Chapter 6**. The isolates from all patients with pneumococcal meningitis in The Netherlands in 1994 were analyzed. The genetic heterogeneity among these penicillin-susceptible strains was substantially higher than that among Dutch penicillin-nonsusceptible isolates. Different capsular types were found among genetically related strains, indicating that horizontal transfer of capsular genes is common among the penicillin-susceptible strains. This is the first study suggesting the frequent occurrence of horizontal transfer of capsular genes among penicillin-susceptible strains. The genetic relatedness within the specific pneumococcal capsular types was highly variable. The genetic heterogeneity was much higher among isolates of capsular types 3, 7F, 9V and 14 than among isolates of other capsular types i.e. 6A, 6B, 18C, 19F and 23F. We further studied the molecular epidemiological characteristics of capsular type 3 pneumococci. Type 3 is considered as the most virulent capsular type and is commonly associated with invasive disease in adults. Epidemiologically unrelated capsular type 3 isolates from four distinct countries were genetically more related than those of other capsular types, suggesting that capsular type 3 has evolved only recently or has remained unchanged over large periods.

The high frequency of capsular gene transfer among pneumococci as described in Chapters 2, 3, 4 and 6 may have consequences for the long-term efficacy of pneumococcal conjugate vaccines. Recently, it was demonstrated that the use of conjugate vaccines with a restricted number of capsular polysaccharide types results in a shift in the distribution of capsular types towards those pneumococcal polysaccharide types that are not present in the vaccine. The horizontal transfer of capsular genes may enhance such a shift. In this respect, capsular

switch leading to the acquisition of non-vaccine capsular types by penicillin- and multidrug-resistant pneumococcal strains forms a major threat. In **Chapter 7**, the effect of immune-protective antibodies raised by large-scale vaccination on horizontal transfer of DNA was investigated. To this purpose, the effect of opsonisation of *S. pneumoniae* with capsular antibodies on the horizontal transfer of DNA was studied. Opsonisation did not inhibit DNA uptake. This suggests that horizontal transfer of capsular genes, which is an important escape mechanism of the pathogen, remains a potential threat for the efficacy of conjugate vaccination.

In **Chapter 8**, proteins were identified that are presumed to play a role in the infectious process. The pneumococcus undergoes spontaneous phase variation resulting in opaque and transparent colony forms. Differences in colony opacity have been shown to correlate with differences in virulence: the transparent variants are more capable of colonizing the nasopharynx, whereas the opaque variants show increased virulence during systemic infections. The molecular changes that occur during phase variation were investigated. To this purpose, protein expression patterns of pneumococcal phenotypic variants were compared. Three differentially expressed proteins were identified and characterized. One protein was overexpressed in the opaque phenotype. This protein revealed significant homology with elongation factor Ts (EF-Ts). EF-Ts is essential for the elongation of the polypeptide chain during protein synthesis. We hypothesized that the increased presence of EF-Ts in the opaque variants of *S. pneumoniae* indicates that the opaque variants are metabolically more active, which may explain the rapid invasive growth characteristics of these variants. Two proteins were overexpressed in the transparent variant. One of these proteins was pyruvate oxidase SpxB. This enzyme decarboxylates pyruvate to acetyl phosphate resulting in the release of hydrogen peroxide and carbon dioxide. As a *spxB*-defective pneumococcal mutant has decreased ability to colonize the nasopharynx, our data suggest that SpxB plays an important role in the enhancement of the ability of transparent variants to efficiently colonize the nasopharynx. The second protein overexpressed in transparent variants revealed significant homology with the proteinase maturation protein PrtM of *Lactocobacillus paracasei*, a member of the family of peptidyl-prolyl *cis/trans* isomerases. Proteins belonging to the family of peptidyl-prolyl *cis/trans* isomerases are thought to assist in protein folding by catalyzing *cis/trans* isomerization of peptidyl-prolyl bonds in peptides and proteins. The pneumococcal putative proteinase maturation protein A, PpmA, contains an N-terminal lipoprotein signal sequence, which suggests that PpmA is

membrane-bound. The higher prevalence of PpmA in the transparent phenotype, which is selected for during nasopharyngeal colonization, suggests that PpmA is either directly involved in adherence through maturation of surface proteins with adherence properties or indirectly by the activation of proteases or other secreted proteins.

In **Chapter 9**, pyruvate oxidase was identified to play a role in the bactericidal effect of the pneumococcus on *Haemophilus influenzae* and in the inhibitory effect on the growth of *Moraxella catarrhalis* and *Neisseria meningitidis*. The release of hydrogen peroxide by pyruvate oxidase activity was demonstrated responsible for these effects. *S. pneumoniae* produce approximately the same amount of hydrogen peroxide per gram of total cellular protein as neutrophils during the oxidative burst. The pneumococcus thus generates unusually high amounts of hydrogen peroxide and, thereby, inhibits and/or kills other species that may compete for the same environmental niche in the heavily colonized human nasopharynx. *H. influenzae* was the most sensitive bacterial species among those tested to both the inhibitory and bactericidal effects of the pneumococcus. If a similar effect occurs in vivo, this could at least in part account for the previously noted lower than expected rates of co-infection with *S. pneumoniae* and *H. influenzae* in otitis media and chronic bronchitis. The increased production of hydrogen peroxide associated with the transparent phenotype may, therefore, contribute to its ability to efficiently colonize a host, being in part the result of the ability to suppress local competitors.

In the study described in **Chapter 10**, we isolated a pool of hydrophobic, surface-associated proteins of *S. pneumoniae*. Surface-exposed proteins often play an important role in the interaction between pathogenic bacteria and their host. The protein fraction was able to induce antibodies that facilitated phagocytosis in vitro. Phagocytosis is a major host defense mechanism against pneumococci, and the induction of opsonophagocytic antibodies is presumed to correlate with in vivo protection against *S. pneumoniae* infection. Antibodies raised against three distinct proteins contributed to the opsonophagocytic activity of the serum. Two proteins were the previously characterized pneumococcal surface protein A and oligopeptide-binding lipoprotein AmiA. The third protein was PpmA, the protein first identified in Chapter 8. Immunoelectron microscopy demonstrated that PpmA was associated with the pneumococcal surface. PpmA was shown to elicit species-specific opsonophagocytic antibodies that were cross-reactive to various pneumococcal strains. This antibody cross-reactivity was in line with the limited sequence variation of *ppmA*. The importance of PpmA

for pneumococcal pathogenesis was demonstrated in a mouse pneumonia model. Pneumococcal *ppmA*-deficient mutants showed reduction in virulence. The here reported properties of PpmA indicate its potential for use in multicomponent protein vaccines.

SAMENVATTING

Streptococcus pneumoniae (de pneumokok) is een humaan pathogeen en behoort tot de belangrijkste veroorzakers van invasieve infecties, zoals longontsteking (pneumonie), bloedvergiftiging (sepsis) en hersenvliesontsteking (meningitis). Daarnaast is de pneumokok wereldwijd een van de belangrijkste veroorzakers van middenoorontsteking (otitis media) en voorhoofdsholte ontsteking (sinusitis). Pneumokokken infecties komen het meest voor bij jonge kinderen, ouderen en personen met immuunstoornissen. Tot in de zestiger jaren ging men ervan uit dat alle pneumokokken gevoelig waren voor het antibioticum penicilline. Tijdens de laatste 30 jaar worden wereldwijd echter steeds meer penicilline-resistente pneumokokken geïsoleerd. Resistentie tegen penicilline, met name in combinatie met resistentie tegen andere soorten antibiotica, zou kunnen leiden tot een falen van de huidige therapeutische behandelingen. Daarom wordt veel aandacht besteed aan de preventie van pneumokokken infecties. Verschillende zaken op het gebied van de verspreiding (epidemiologie) van pneumokokken infecties zijn van belang bij het ontwikkelen van goede behandel- en preventie-strategieën. Zo kunnen vier vragen gesteld worden. Hoe verspreidt de bacterie zich binnen de menselijke populatie? Zijn bepaalde subgroepen binnen de pneumokokken populatie sterk vertegenwoordigd? Zo ja, wat zijn de virulentie karakteristieken en de resistentie profielen van deze subgroepen? Zal de pneumokokken populatie veranderen na grootschalig gebruik van vaccins? Het huidige vaccin bestaat uit een mix van de 23 meest voorkomende pneumokokken kapselpolysaccharide typen. Op dit moment zijn 90 verschillende kapselpolysaccharide typen beschreven. Dit 23-valente vaccin heeft de mortaliteit en morbiditeit veroorzaakt door *S. pneumoniae* nauwelijks verminderd. Een belangrijke reden hiervoor is dat kinderen die jonger zijn dan 2 jaar niet in staat zijn voldoende antilichamen te produceren tegen polysaccharide vaccins. De huidige pneumokokken vaccin-strategieën richten zich daarom op het gebruik van conjugaat vaccins. Conjugaat vaccins bestaan uit kapselpolysacchariden gekoppeld aan een zeer immunogeen dragereiwit. De antilichaam vorming tegen de polysacchariden wordt hierdoor T-cell afhankelijk in plaats van T-cell onafhankelijk, dat wil zeggen dat de antilichaam respons tegen polysacchariden verhoogd wordt en een immunologisch geheugen opgewekt wordt. Een nadeel van conjugaat vaccins is dat het slechts een beperkt aantal verschillende polysaccharide typen kan bevatten. De laatste jaren wordt veel onderzoek gedaan naar de rol van pneumokokken eiwitten in het ziekteproces en het vermogen van deze eiwitten om een beschermende immuunrespons op te wekken. Eiwitten die betrokken zijn bij het ziekteproces van pneumokokken infecties worden interessant geacht voor toepassing in toekomstige conjugaat vaccins of voor de ontwikkeling van veelcomponenten eiwit vaccins.

Het doel van mijn onderzoeksproject was het bestuderen van zowel moleculair epidemiologische aspecten van *S. pneumoniae* als de identificatie van pneumokokken eiwitten die betrokken zijn bij het ziekteproces.

Drie moleculair epidemiologische studies zijn uitgevoerd om de verspreiding van penicilline-ongevoelige pneumokokken isolaten te onderzoeken. **Hoofdstuk 2** beschrijft een internationaal onderzoek naar de verspreiding van penicilline-ongevoelige isolaten afkomstig uit 16 verschillende landen uit de hele wereld. De twee meest voorkomende genetische types komen overeen met een Spaans pandemische kloon 23F en een Spaans-Frans internationale kloon 9V. De wereldwijde verspreiding van deze klonen is duidelijk aangetoond, want ze zijn gevonden in respectievelijk 10 en zes landen.
Verschillende genetische groepen bevatten twee of meer kapseltypen. Hieruit blijkt het frequente voorkomen van horizontale overdracht van kapselgenen. Daarnaast zijn identieke penicilline-bindende eiwit (PBP) gentypen gevonden bij verschillende genetische typen. Hieruit blijkt dat horizontale DNA overdracht van penicilline resistentie genen vaak voorkomt. De overgrote meerderheid van de stammen, behorende tot de pandemische klonen 23F en 9V, hebben één en hetzelfde PBP type. Wij veronderstellen dat de klonen 23F en 9V verantwoordelijk zijn voor de wereldwijde toename van penicilline resistentie, en dat deze klonen een belangrijk genetisch reservoir vormen voor gevoelige pneumokokken om penicilline resistentie te verwerven.

Hoofdstuk 3 beschrijft het epidemiologisch karakter van de toename van penicilline-ongevoelige pneumokokken in Polen. Er zijn vier genetisch verschillende groepen aangetoond binnen een collectie penicilline-ongevoelige stammen, die zijn geïsoleerd in 16 verspreid over het land gelegen steden in 1995 en 1996. Twee groepen komen overeen met de pandemische klonen 23F en 9V, die zich recentelijk over de wereld hebben verspreid. De twee andere genetische groepen met kapseltypen 23F en 6V zijn met name belangrijk in de onderzochte collectie van Poolse penicilline-ongevoelige pneumokokken isolaten. De twee laatste groepen vinden hun oorsprong waarschijnlijk in Polen, omdat de groepen niet overeen komen met genetische typen van penicilline-ongevoelige isolaten die verzameld zijn in 15 andere landen.

Het epidemiologisch karakter van de explosieve toename van penicilline-ongevoelige pneumokokken in Thailand is onderzocht in **Hoofdstuk 4**. De belangrijkste multiresistente

pneumokokken klonen in Thailand zijn de pandemische klonen 23F en 9V. Daarom veronderstellen wij dat deze pandemische klonen primair verantwoordelijk zijn voor de toename in het voorkomen van pneumokokken penicilline resistentie in Thailand.

De moleculaire mechanismen van penicilline resistentie in Nederland zijn in detail bestudeerd in **Hoofdstuk 5**. Pneumokokken isolaten brengen zes penicilline-bindende eiwitten (PBPs) tot expressie. Veel penicilline-ongevoelige isolaten zijn veranderd in alleen PBP1a, PBP2b and PBP2x. In deze studie hebben we fragmenten gekarakteriseerd van de genen *pbp1a*, *pbp2b* en *pbp2x* van Nederlandse klinische isolaten. De pneumokokken stammen zijn geselecteerd op basis van verschillen in hun restrictie fragment lengte polymorfisme (RFLP) patroon van de genen *pbp1a*, *pbp2b* en *pbp2x*. Classificatie van de *pbp* fragmenten op basis van bepaling van de base volgorde kwam goed overeen met de classificatie op basis van RFLP analyse. De mutaties in de *pbp* base volgorde van de Nederlandse isolaten blijken allen overeen te komen met de *pbp* mutaties zoals beschreven bij penicilline-ongevoelige isolaten afkomstig uit andere landen. Deze waarneming bevestigt de veronderstelling dat meerdere klonen van penicilline-resistente pneumokokken naar Nederland zijn geïmporteerd en over het land zijn verspreid. Interessant is dat daarnaast ook nieuwe combinaties van mosaïc structuren geïdentificeerd zijn. Dit laatste duidt op DNA overdracht van *pbp* fragmenten tussen penicilline-ongevoelige isolaten.

De moleculair epidemiologische karakteristieken van penicilline-gevoelige Nederlandse *S. pneumoniae* isolaten zijn bestudeerd in **Hoofdstuk 6**. De isolaten van alle pneumokokken meningitis patiënten in Nederland in 1994 zijn geanalyseerd. De genetische diversiteit binnen deze penicilline-gevoelige stammen was substantieel hoger dan binnen Nederlandse penicilline-ongevoelige isolaten. Binnen genetisch gerelateerde stammen zijn verschillende kapseltypen gevonden. Dit duidt op het voorkomen van horizontale overdracht van kapselgenen binnen penicilline-gevoelige isolaten. Voor zover wij weten is dit de eerste studie waarin het frequente voorkomen van horizontale overdracht van kapselgenen binnen penicilline-gevoelige stammen gesuggereerd wordt. De genetische verwantschap binnen stammen met specifieke pneumokokken kapseltypen was erg gevarieerd. De genetische verscheidenheid was veel groter tussen isolaten met de kapseltypen 6A, 6B, 18C, 19F en 23F dan tussen isolaten met de kapseltypen 3, 7F, 9V en 14. De moleculair epidemiologische karakteristieken van isolaten met kapseltype 3 zijn uitgebreider bestudeerd. Kapseltype 3 wordt gezien als het meest virulente kapseltype en wordt vaak geassocieerd met invasieve

infecties in volwassenen. Epidemiologisch niet-gerelateerde kapseltype 3 isolaten, afkomstig uit vier verschillende landen, waren genetisch meer verwant dan isolaten met andere kapseltypen. Dit duidt op een mogelijk recent ontstaan van kapseltype 3 isolaten of op genetische stabiliteit gedurende lange tijd.

Het frequente voorkomen van overdracht van kapselgenen zoals onder andere beschreven in de hoofdstukken 2, 3, 4 en 6 kan consequenties hebben voor de effectiviteit van pneumokokken conjugaat vaccins op lange termijn. Recentelijk is aangetoond dat het gebruik van conjugaat vaccins met een beperkt aantal kapselpolysaccharide typen leidt tot een toename in het voorkomen van kapseltypen die niet in het vaccin opgenomen zijn. Horizontale overdracht van kapselgenen zou deze toename kunnen versterken. Men name het verkrijgen van niet-vaccin kapseltypen door penicilline- en multi-resistente pneumokokken vormt een grote bedreiging. In **Hoofdstuk 7** wordt het effect van vaccinatie op horizontale overdracht van DNA bestudeerd door de efficiëntie van DNA-opname te bepalen na binding van antilichamen gericht tegen het pneumokokken kapsel. Antilichaam-binding heeft geen (remmend) effect op de opname van DNA. Daarom is het waarschijnlijk dat horizontale overdracht van kapselgenen, dat een belangrijk ontsnappingsmechanisme voor de ziekteverwekker is, een potentiële bedreiging blijft voor de effectiviteit van vaccinatie met conjugaat vaccins.

In **Hoofdstuk 8** zijn eiwitten geïdentificeerd die geacht worden een rol te spelen in het ziekteproces. De pneumokok ondergaat spontaan fase variatie, waarbij ondoorschijnende (opake) kolonievormen en heldere (transparante) kolonievormen in elkaar overgaan. Verschil in de doorzichtigheid van de kolonies correleert met verschillen in virulentie: de transparante variant is beter in staat om de neus-keelholte te koloniseren, terwijl de opake variant een verhoogde virulentie vertoont bij systemische infecties. De moleculaire veranderingen die plaatsvinden tijdens deze fase variatie zijn onderzocht, door de eiwit expressie patronen van de twee fenotypische varianten met elkaar vergelijken. De eiwitten die verschillend tot expressie komen, zijn geïdentificeerd en gekarakteriseerd. Een eiwit dat hoger tot expressie komt in het opake fenotype blijkt homoloog te zijn met elongatie factor Ts (EF-Ts). EF-Ts is essentieel voor de verlenging van de polypeptide keten tijdens eiwit synthese. Wij veronderstellen dat de grotere aanwezigheid van EF-Ts in de opake variant duidt op een hogere metabolische activiteit van deze variant. Dit zou de snelle invasieve groei karakteristieken kunnen verklaren van de opake variant. Twee eiwitten zijn in grotere

hoeveelheden aanwezig in de transparante variant. Een van deze eiwitten is pyruvaat oxidase, SpxB. Dit enzym decarboxyleert pyruvaat tot acetylfosfaat wat leidt tot het vrijkomen van waterstofperoxide en koolstofdioxide. Omdat een defect in *spxB* leidt tot een verminderd vermogen van de pneumokok om de neus-keelholte te koloniseren, suggereren deze data dat pyruvaat oxidase een belangrijke rol speelt in het vermogen van transparante varianten om de neus-keelholte te koloniseren. Het tweede eiwit dat hoger tot expressie komt in de transparante variant is homoloog aan het proteinase maturatie eiwit PrtM van *Lactobacillus paracasei*. PrtM is lid van de familie van peptidyl-prolyl *cis/trans* isomerases. Gedacht wordt dat eiwitten die tot deze familie behoren de vouwing van eiwitten vergemakkelijken door de *cis/trans* isomerisatie van peptidyl-prolyl verbindingen in peptiden en eiwitten te katalyseren. Het vermeende pneumokokken proteinase maturatie eiwit A, PpmA, bevat een N-terminale uiteinde dat wijst op membraan binding. De verhoogde productie van PpmA in het transparante fenotype, dat tijdens de kolonisatie van de neus-keelholte geselecteerd wordt, suggereert dat PpmA direct betrokken is bij de adherentie door het correct vouwen van oppervlakte eiwitten met adhesie eigenschappen of indirect door activatie van proteases of andere gesecreteerde eiwitten.

In **Hoofdstuk 9** is aangetoond dat pyruvaat oxidase een rol speelt in het afdoden van *Haemophilus influenzae* en het remmen van de groei van *Moraxella catarrhalis* en *Neisseria meningitidis* door de pneumokok. Het vrijkomen van waterstofperoxide door pyruvaat oxidase activiteit blijkt verantwoordelijk voor deze effecten. *S. pneumoniae* produceert ongebruikelijk grote hoeveelheden waterstofperoxide vergelijkbaar met fagocyterende neutrofielen. De pneumokok remt en/of doodt daarmee andere bacterie-soorten die wedijveren om dezelfde niche in de stevig gekoloniseerde humane neus-keelholte. *H. influenzae* was het meest gevoelig van de geteste bacterie-soorten voor de remmende en afdodende werking van de pneumokok. Mogelijk verkaart dit fenomeen de minder dan verwachte mate van co-infectie van *S. pneumoniae* en *H. influenzae* in otitis media en chronische bronchitis. De verhoogde productie van waterstofperoxide door het transparante fenotype, met als gevolg het onderdrukken van lokale concurrenten, zou daarom bij kunnen dragen aan zijn vermogen om de gastheer efficiënt te kunnen koloniseren.

Eiwitten die aan het oppervlak liggen spelen vaak een belangrijke rol in de interactie tussen bacterie en gastheer. In de studie beschreven in **Hoofdstuk 10** hebben we een fractie van hydrofobe, oppervlakte-geassocieerde eiwitten van *S. pneumoniae* geïsoleerd. De eiwitfractie

was in staat antilichamen op te wekken. Deze antilichamen vergemakkelijkten fagocytose in vitro. Fagocytose is een belangrijk afweermechanisme van de gastheer tegen de pneumokok. De fagocytose capaciteit van antilichamen wordt geacht te correleren met in vivo bescherming tegen *S. pneumoniae* infecties. De antilichamen, die opgewekt zijn tegen drie geïsoleerde eiwitten, droegen bij aan de fagocytose capaciteit van het serum. Twee eiwitten bleken de eerder gekarakteriseerde eiwitten "pneumococcal surface protein A" en "oligopeptide binding lipoprotein AmiA" te zijn. Het derde eiwit was PpmA: het eiwit dat eerder geïdentificeerd is in hoofdstuk 8. Immunoelectronen microscopie heeft aangetoond dat PpmA geassocieerd is aan het oppervlak van de pneumokok. PpmA blijkt in staat te zijn soort-specifieke antilichamen met fagocytose capaciteit op te wekken, die kruisreageren met verschillende pneumokokken stammen. Deze kruisreactiviteit komt overeen met de beperkte variatie in base volgorde van *ppmA* van verschillende pneumokokken stammen. Het belang van PpmA in het ziekteproces van pneumokokken infecties is aangetoond in een muis model voor longontsteking. Pneumokokken mutanten met een deficiëntie in *ppmA* bleken minder virulent. De gevonden karakteristieken van PpmA maken PpmA een interessant eiwit voor het gebruik in meercomponenten eiwit vaccins.

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CURRICULUM VITAE

Karin Overweg werd op 4 april 1970 geboren in Waalwijk. In 1988 behaalde zij het VWO diploma aan de Lorenz Scholengemeenschap in Arnhem. In datzelfde jaar begon zij met de studie Biologie aan de Katholieke Universiteit in Nijmegen (K.U.N.). Deze studie rondde zij in augustus 1994 af. Haar stages bracht zij door bij de afdeling Moleculaire Plantenfysiologie, K.U.N. (Dr. M.T. Oldenhof, Dr. J.A. Schrauwen) en de afdeling Moleculaire Biologie, K.U.N. (Dr. H. J. Kraft; Dr. N.H. Lubsen). Als extra stage werd via de afdeling Dierfysiologie, K.U.N. (Prof. Dr. G.J.A. Martens) bij de afdeling Biotechnology (Dr. B.A.P. Chaudhuri) van Ciba Geigy in Basel onderzoek verricht. Van mei 1995 tot augustus 1999 was zij werkzaam als assisistent in opleiding bij de afdeling Kindergeneeskunde van de Erasmus Universiteit in Rotterdam. Gedurende deze periode werd onder begeleiding van Dr. P.W.M. Hermans en Prof. Dr. R. de Groot het in dit proefschrift beschreven onderzoek verricht. Vanaf juli 2000 is zij werkzaam als postdoc bij het Institute of Food Research in Norwich, Engeland.

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