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Genetic Relationships between Clinical Isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: Characterization of "Atypical" Pneumococci and Organisms Allied to *S. mitis* Harboring *S. pneumoniae* Virulence Factor-Encoding Genes

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The oral streptococcal group (mitis phylogenetic group) currently consists of nine recognized species, although the group has been traditionally difficult to classify, with frequent changes in nomenclature over the years. The pneumococcus (*Streptococcus pneumoniae*), an important human pathogen, is traditionally distinguished from the most closely related oral streptococcal species *Streptococcus mitis* and *Streptococcus oralis* on the basis of three differentiating characteristics: optochin susceptibility, bile solubility, and agglutination with antipneumococcal polysaccharide capsule antibodies. However, there are many reports in the literature of pneumococci lacking one or more of these defining characteristics. Sometimes called "atypical" pneumococci, these isolates can be the source of considerable confusion in the clinical laboratory. Little is known to date about the genetic relationships of such organisms with classical *S. pneumoniae* isolates. Here we describe these relationships based on sequence analysis of housekeeping genes in comparison with previously characterized isolates of *S. pneumoniae*, *S. mitis*, and *S. oralis*. While most pneumococci were found to represent a closely related group these studies identified a subgroup of atypical pneumococcal isolates (bile insoluble and/or "acapsular") distinct from, though most closely related to, the "typical" pneumococcal isolates. However, a large proportion of isolates, found to be atypical on the basis of capsule reaction alone, did group with typical pneumococci, suggesting that they have either lost capsule production or represent as-yet-unrecognized capsular types. In contrast to typical *S. pneumoniae*, isolates phenotypically identified as *S. mitis* and *S. oralis*, which included isolates previously characterized in taxonomic studies, were genetically diverse. While most of the *S. oralis* isolates did fall into a well-separated group, *S. mitis* isolates did not cluster into a well-separated group. During the course of these studies we also identified a number of potentially important pathogenic isolates, which were frequently associated with respiratory disease, that phenotypically and genetically are most closely related to *S. mitis* but which harbor genes encoding the virulence determinants pneumolysin and autolysin classically associated with *S. pneumoniae*.

Streptococcus pneumoniae is a common and important human pathogen associated with pneumonia, septicaemia, meningitis, and otitis media. A number of distinct species of naturally transformable viridans or oral streptococci, which are closely related to *S. pneumoniae*, have now been identified, although the taxonomy and classification of these organisms has long been considered difficult (47). The most closely related species on the basis of 16S rRNA sequence are *Streptococcus oralis* and *Streptococcus mitis*, which share over 99% sequence identity with *S. pneumoniae*, although DNA-DNA similarity values for the entire chromosome are estimated to be less than 60% (28). *S. oralis* and *S. mitis* are usually considered to be commensals of the human oral cavity, but in recent years it has become clear that members of these species can be important pathogens. Oral streptococci, including *S. mitis* and *S. oralis*, are associated with bacterial endocarditis, especially in patients with prosthetic valves (16). In addition, *S. mitis* and *S. oralis* are now recognized as frequent causes of infection in immunocompromised patients, particularly immediately after tissue transplants, and in neutropenic cancer patients (5, 8, 9, 33).

Four phenotypic characteristics are classically used in the diagnostic laboratory for the presumptive identification of *S. pneumoniae*: colony morphology, optochin sensitivity, bile solubility, and agglutination with antipneumococcal polysaccharide capsule antibodies. Although their colony morphology can be very similar, nonpneumococcal oral streptococci are classically optochin resistant and bile insoluble and do not react with antipolysaccharide antibodies. It is considered important for the laboratory to differentiate between *S. pneumoniae* and other alpha-hemolytic oral streptococci, notably *S. mitis* and *S. oralis*, since misidentification may influence diagnosis and treatment. While these conventional methods allow identification of the majority of pneumococcal isolates, presumptive *S. pneumoniae* isolates may produce atypical reactions in one or more of the standard tests, and other alpha-hemolytic streptococci may give positive reactions in these tests, leading to difficulties in identification. For example, there are many ob-

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servations of optochin-resistant pneumococci (1, 30, 37, 38), bile-insoluble *S. pneumoniae* isolates have been reported (15, 21, 36), and nontypeable, unencapsulated pneumococci have historically been reported to comprise 2% of pneumococci isolated from normally sterile sites (10) and up to 20% of conjunctival isolates (22).

Despite these problems, most diagnostic laboratories still use these conventional identification techniques. A commercially available DNA probe test (AccuProbe Culture Identification Kit for *S. pneumoniae*) has been reported to show specificity and sensitivity of 100% for pneumococci (13, 14), but it is prohibitively expensive for routine diagnostic use (36). The use of PCR in diagnostic tests, targeting genes considered to be specific targets for the pneumococcus, has been investigated. Two attractive targets for such studies have been genes encoding the putative virulence factors pneumolysin (*ply*) and the major autolysin (*lytA*) (25, 40, 41, 43). Such PCR-based diagnostics, while offering some promise, are not, as yet, used frequently in the routine microbiology laboratory setting.

Here we describe the preliminary characterization of a group of "atypical" presumptive isolates of *S. pneumoniae* which show aberrant reactions to bile and/or do not react with antipneumococcal polysaccharide antibodies. We have used sequencing of housekeeping genes to investigate the genetic background of these organisms in comparison to a selection of "typical" pneumococci and both previously characterized and clinical isolates of *S. oralis* and *S. mitis*. In addition, we describe a second group of atypical isolates identified during the course of this study. These organisms appear to be genetically and phenotypically related to *S. mitis* but harbor genes encoding the virulence factors autolysin and pneumolysin, which are normally associated with pneumococci. Interestingly, many of these isolates were disease associated. The relationships of these organisms to other well-characterized oral streptococci and a preliminary characterization of the genes encoding autolysin and pneumolysin in these organisms are described here.

MATERIALS AND METHODS

Strains. A complete list of strains used in this study of genetic diversity is provided in Table 1. Isolates were routinely cultured on brain heart infusion (BHI) agar supplemented with 5% (vol/vol) sheep's blood at 37°C and 5% CO_2. The origins of typical pneumococcal strains used in the determination of *lytA* diversity have been described elsewhere (44).

Strain phenotypes. Phenotypes were determined by the standard criteria used in the distinction of oral streptococci: optochin sensitivity, bile solubility, and latex agglutination-capsular serotyping.

(i) Optochin sensitivity. For the determination of optochin sensitivity, each isolate was cultured in 5 ml of Todd-Hewitt broth. A 1:10 dilution was prepared from the broth culture and used to flood a 5% horse blood agar plate. A 5-μg optochin disk (Mast Diagnostics) was placed on the dried plate which was incubated at 37°C for 18 h. Zone size diameters were then measured (in millimeters) and, as stated by the manufacturer, isolates with zones of >15 mm were considered to be sensitive. Isolates showing zones of 10 to 14 mm were considered to be of reduced sensitivity (illustrated by S/R in Table 1).

(ii) Bile solubility. Bile solubility was determined by growing organisms in two 5-ml volumes of Todd-Hewitt broth supplemented with 0.5 ml of 20% (wt/vol) glucose. After centrifugation at 3,000 rpm for 20 min the supernatants were discarded, both cell pellets were resuspended in 2.5 ml of phosphate-buffered saline (PBS), and 0.5 ml of 10% (wt/vol) sodium deoxycholate was added to one of the suspensions. These were incubated at 37°C for 15 to 30 min. Complete lysis within 30 min of incubation was indicative of a positive result.

(iii) Latex agglutination and capsular serotyping. Latex agglutination was performed with organisms grown in 5 ml of Todd-Hewitt broth. The centrifuged deposit was tested with the Slidex Pneumo kit (Biomerieux), which comprises a latex suspension sensitized with antisera to 83 pneumococcal capsular serotypes. The test was performed according to the manufacturer's instructions. Capsular serotyping was performed on cultures grown in 4.5 ml of Todd-Hewitt broth, supplemented with 0.5 ml of 20% (wt/vol) glucose. The cultures were centrifuged at 3,000 rpm for 20 min, and the supernatants were discarded. The pellet (antigen suspension) was vortexed, and slide agglutination was performed against the capsular typing sera (Statens Serum Institut). So-called rough nontypeable strains autoagglutinated with the latex reagent and therefore could not be subject to capsular serotyping, while smooth nontypeable strains did not autoagglutinate but did not react with any of the pooled capsular typing sera.

(iv) DNA probe. The Accuprobe *Streptococcus pneumoniae* Culture Identification Test (Gen-Probe, San Diego, Calif.) was used to screen selected isolates. The test is based on the detection of a specific rRNA-encoding sequence found in *S. pneumoniae* and was performed according to the manufacturer's instructions with four colonies from an overnight culture on 5% blood agar. Any isolate giving a reading of >1,500 photometric light units (PLU) was considered positive, and a reading below this level was considered negative. The test was repeated if the value fell to 1,200 to 1,499 PLU.

(v) Biotyping. Biotyping was performed using the rapid ID32 Strep (Biomerieux UK, Ltd.). Cultures were grown anaerobically on blood agar at 37°C for 18 h, and suspensions were prepared according to the manufacturer's instructions. The resulting API profile was used to obtain an identification with the Apilab computer key. Additional tests were performed to confirm the identification. These included the catalase reaction, which was performed on an 18-h culture grown on serum glucose agar in 5% CO_2, and the test for dextran-levan production (12).

(vi) Assay of hemolytic activity. Assays of hemolytic activity associated with strains were performed by using a modification of the method described by Benton et al. (6). Cultures were grown to an optical density at 600 nm of 0.4, and assays of both cytoplasmic and extracellular hemolytic activity were performed. Four milliliters of culture was centrifuged for 10 min at 3,000 rpm. The supernatant was retained for analysis of extracellular haemolytic activity, while the pellet was lysed by incubation in 400 μl of lysis buffer (0.01% [wt/vol] sodium dodecyl sulfate, 0.1% [wt/vol] sodium deoxycholate, 0.015 M sodium citrate) at 37°C for 30 min. Hemolytic titers obtained from the supernatant fractions were multiplied by 10 in order to represent numbers of pneumococci comparable with those used to calculate the cytoplasmic titers. In brief, 160 μl of dithiothreitol (DTT) buffer (10 mM DTT–0.1% [wt/vol] bovine serum albumin dissolved in PBS) was added to 96-well V-bottom plates. To the first well of a column 80 μl of culture medium (BHI) or lysed streptococci was added and serially diluted 1:3 across the plate. Control wells consisted of a dilution series of lysate buffer in DTT and BHI alone. Eighty microliters of 2% (vol/vol) horse blood, washed three times and diluted in PBS, was added to all wells. The plates were incubated at 37°C for 30 min and then centrifuged for 10 min at 2,000 rpm. The plates were scored for complete lysis of horse blood, as indicated by the absence of a pellet of cells. The specificity of the assay for pneumolysin was demonstrated by including a D39 derived mutant in which *ply* had been disrupted by the insertion of an erythromycin resistance cassette and the isogenic parent strain in the assay.

Preparation of chromosomal DNA. Chromosomal DNA was prepared from all isolates as described previously (45).

Sequence analysis of housekeeping genes. Fragments of DNA corresponding to the housekeeping genes *hexB* (DNA mismatch repair protein), *recP* (transketolase), and *xpt* (xanthine phosphoribosyltransferase) were amplified by PCR using the primer sets *hexB*up (5′-CCATTGACGCGGGCTCTA-3′) and *hex*Bdn (5′-CCTGAATACGTCGGAACATCTTT-3′), *recP*up (5′-ACCGCGACCGCT TTATTCTTTC-3′) and *recP*dn (5′-ATGCTGACTACGCGGGATTTTTC-3′), and *xpt*up (5′-GAAATTATTAGAAGAA/GCGCATC-3′) and *xpt*dn (5′-TTAG AGATCTGCCTCCA/TTA/GAA-3′), respectively. PCR was performed under standard conditions with 32 cycles of 95°C for 1 min, X°C for 1 min, and 72°C for 1 min, where X°C represents an annealing temperature appropriate for the particular primer set used. Fragments were purified through Qiagen PCR purification columns and sequenced directly by using the same primers and an ABI 373 automated sequencing system. Sequences of 395bp (*xpt*up), 285bp (*hexB*up), 351bp (*hexB*dn), 327bp (*recP*up), and 339bp (*recP*dn) were obtained from each isolate and used in the analysis presented here.

Phylogenetic analysis. Preliminary analysis and alignment of sequences was performed in DNAStar. Phylogenetic analysis was performed by using the MEGA suite of programs (31). Dendrograms were constructed by the neighbor-joining method with the Jukes-Cantor correction. The percentage bootstrap confidence levels of internal branches, as defined for MEGA (31), were calculated from 500 random resamplings of the original sequence data with replacement.

Screening of isolates for pneumolysin and autolysin genes. All isolates were screened for the presence of the pneumolysin (*ply*), major autolysin (*lytA*), and atypical autolysin (*lytA*101) encoding genes by PCR with primer sets *ply*up-*ply*dn and *lytA*up-*lytA*dn as described previously (46) or, in the case of *lytA*101, the primer *lytA*up in combination with a novel specific reverse primer *lytA*101dn (5′-CTACTTCATCGTAATCAAACCGTCAGGTTC-3′). The specificity of PCR products was confirmed by probing with digoxigenin-labeled *ply* and *lytA* fragments according to manufacturer's instructions following capillary transfer to a nylon membrane. Probes were obtained after PCR by using the same primer sets, cloning, and confirmatory sequencing of *lytA* and *ply* from *S. pneumoniae* R6 and *lytA*101 from *S. pneumoniae* 101/87.

Nucleotide sequence accession numbers. All sequences obtained in this study have been deposited in EMBL. The housekeeping gene fragments have been assigned the accession numbers AJ240606 to AJ240674 and AJ390850 to AJ391094 and the *lytA* sequence accession numbers AJ252190 to AJ252196.

TABLE 1. Characteristics of strains examined in this study

Strain no.	Other ID	Identification[a]	Origin[b]	Site of isolation[c]	Latex agglutination/serotype[d]	Optochin	Bile solubility	Gen-Probe[e]	Presence of[f]:			Source or reference[g]
									lytA	*lytA101*	*ply*	
Group 1 (typical *S. pneumoniae*)												
PC15	1011	*S. pneumoniae*	UK	Throat	23F	S	+	+	+	−	+	35
11B	912	*S. pneumoniae*	UK	Throat	15B	S	+	+	+	−	+	35
PC19	1012	*S. pneumoniae*	UK	Throat	35A	S	+	+	+	−	+	35
CL1 (E226)	355	*S. pneumoniae*	Uruguay	Blood	14	S	+	ND	+	−	+	35
CL26 (Sp8)	30	*S. pneumoniae*	Spain	Blood	13	S	+	ND	+	−	+	35
R6		*S. pneumoniae*	US		r	S	+	+	+	−	+	2
44A	951	*S. pneumoniae*	UK	Throat	6A	S	+	+	+	−	+	35
CL10 (KD12)	312	*S. pneumoniae*	Kenya	Throat	7F	S	+	ND	+	−	+	35
86013	873	*S. pneumoniae*	Kenya	Throat	8	S	+	+	+	−	+	35
1A	900	*S. pneumoniae*	UK	Throat	1	S	+	+	+	−	+	35
CL13 (Sp9)	555	*S. pneumoniae*	Spain	Blood	8	S	+	ND	+	−	+	35
Group 2a (*S. oralis* and *S. mitis* characterized in previous taxonomic studies)[h]												
NCTC 11427	542	*S. oralis*^{T}			−	R	−	ND	−	−	−	4, 29
NCTC 7864	627/571	*S. oralis*			−	R	−	−	−	−	−	4, 29
OPA1	626	*S. oralis*			−	R	−	ND	−	−	−	4
PP53	619	*S. oralis*			−	R	−	ND	−	−	−	4
HV51	621	*S. mitis*			−	R	−	ND	−	−	−	4
NCTC 10712	567	*S. mitis*			−	R	−	−	−	−	−	4, 29
NS51T	620	*S. mitis*^{T}			−	R	−	ND	−	−	−	4
K208	622	*S. mitis*			−	R	−	ND	−	−	−	4
OS51	624	*S. mitis*			−	R	−	ND	−	−	−	4
Group 2b (commensal and/or clinical isolates of *S. oralis* and *S. mitis*)												
AR37	1078	*S. oralis*	UK	Blood, endocarditis	−	R	−	ND	−	−	−	D. Beighton
COL21	PN93/447	*Streptococcus* sp.	UK	Eye, eye disease	−	R	−	−	−	−	−	This study
COL25	PN93/1003	*S. oralis*	UK	Blood, endocarditis	−	R	−	−	−	−	−	This study
AR5	1072	*S. oralis*	UK	Blood, endocarditis	−	R	−	ND	−	−	−	D. Beighton
AR13	1076	*S. oralis*	UK	Endocarditis	−	R	−	ND	−	−	−	D. Beighton
AC1372	1039	*S. oralis*	UK	Blood, endocarditis	−	R	−	ND	−	−	−	D. Beighton
C17	1073	*S. oralis*	UK	Neutropenia	−	R	−	ND	−	−	−	D. Beighton
COL19	PN93/800	*S. oralis*	UK	Blood	−	R	−	−	−	−	−	This study
COL22	PN93/1264	*Streptococcus* sp.	UK	NK	−	R	−	−	−	−	−	This study
M1	T6(2) 10	*S. mitis*	UK	Normal flora	−	R	−	ND	−	−	−	D. Beighton
M3	1304/16	*S. mitis*	UK	Normal flora	−	R	−	ND	−	−	−	D. Beighton
M4	T6(2) 11	*S. mitis*	UK	Normal flora	−	R	−	ND	−	−	−	D. Beighton
M2	T6(3) 12	*S. mitis*	UK	Normal flora	−	R	−	ND	−	−	−	D. Beighton
CL22	103	*S. mitis*	Spain	Lower respiratory tract	−	R	−	ND	−	−	−	This study
AC1374	1040	*S. mitis*	UK	Blood culture	−	R	−	ND	−	−	−	D. Beighton
AC1362	1042	*S. oralis*	UK	Blood culture	−	R	−	ND	−	−	−	D. Beighton
Group 2c (atypical oral streptococci)												
COL18	PN93/454	*Streptococcus* sp.	UK	Sputum, chest infection	−	S	−	−	+	−	+	This study
COL15	PN92/1139	*S. mitis*	UK	Sputum, chest infection	−	S	−	−	+	−	+	This study
COL16	PN93/952	*Streptococcus* sp.	UK	Bronchial lavage, pneumonia, HIV$^+$	−	S	−	−	+	−	+	This study
806		*S. mitis*	UK	NK	−	R	−	−	+	−	+	S. Gillespie
COL28	PN93/918	*Streptococcus* sp.	UK	Sputum, respiratory infection	−	R	−	−	+	−	+	This study
COL20	PN93/776	*Streptococcus* sp.	UK	NK	−	S/R	−	−	+	−	+	This study
COL24	PN93/656	*Streptococcus* sp.	UK	Sputum	−	S/R	−	−	+	−	+	This study
COL17	PN91/2745	*Streptococcus* sp.	UK	NK	−	R	−	−	+	+	+	This study
764		*S. mitis*	UK	NK	−	R	−	−	−	−	+	S. Gillespie
Group 3 (putative atypical pneumococci)												
1916	PN97/3197	*S. pneumoniae*	UK		r	S	+	ND	+	−	+	This study
COL1	PN92/1207	*S. pneumoniae*	UK	NK	r	S	+	+	+	−	+	This study
COL3	PN92/944	*S. pneumoniae*	UK	Eye, eye disease	r	S	+	+	+	−	+	This study
COL6	PN93/779	*S. pneumoniae*	UK	Blood	r	S	+	+	+	−	+	This study

Continued on following page

TABLE 1—*Continued*

Strain no.	Other ID	Identification[a]	Origin[b]	Site of isolation[c]	Latex agglutination/serotype[d]	Optochin	Bile solubility	Gen-Probe[e]	Presence of[f]:			Source or reference[g]
									lytA	*lytA101*	*ply*	
COL5	PN93/832	*S. pneumoniae*	UK	Blood	r	S/R	+	+	+	−	+	This study
COL7	PN93/356	*S. pneumoniae*	UK	Nasal, respiratory infection	r	S/R	+	+	+	−	+	This study
COL8	PN93/707	*S. pneumoniae*	UK	Sputum	−	S/R	+	+	+	−	+	This study
COL11	R93/688	*S. pneumoniae*	UK	CSF, meningitis	−	S	+	+	+	−	+	This study
COL12	PN93/950	*S. pneumoniae*	UK	Blood	−	S	+	+	+	−	+	This study
COL14	PN93/789	*S. pneumoniae*	UK	Blood, pneumonia	−	S	+	+	+	−	+	This study
COL9	PN93/904	*S. pneumoniae*	UK	Blood	−	S/R	+	+	+	−	+	This study
X158		*Streptococcus* sp.			r	S	−	+	+	−	+	This study
COL27	PN93/135	*Streptococcus* sp.	UK	Sputum, chest infect; HIV^+	−	S/R	−	+	+	−	+	This study
COL26	PN93/403	*Streptococcus* sp.	UK	Synovial fluid, septic arthritis	r	R	−	+	+	−	+	This study
101/87		*S. pneumoniae*[h]	Spain	Blood, pneumonia	−	R	−	ND	−	+	+	15
86027	874	*Streptococcus* sp.	Kenya	Throat	−	S	−/+	+	+	−	+	35

[a] Unless stated otherwise, identities are based on the phenotypic tests described in this table in combination with API biotyping and additional biochemical tests when necessary. Where strain profiles were not consistent with any one species or where discordant identities were obtained on separate occasions, strains are not given a species designation. T, type strain.

[b] UK, United Kingdom.

[c] NK, not known; CSF, cerebrospinal fluid; HIV, human immunodeficiency virus.

[d] r, strains which autoagglutinated when latex agglutination-capsular serotyping was performed (rough, nontypeable strains); −, no results against capsular typing sera (smooth, nontypeable strains).

[e] ND, not done.

[f] The presence of *lytA*, *lytA101*, and *ply* was determined by PCR and confirmatory blotting.

[g] D. Beighton, Joint Microbiology Research Unit, Guy's, King's, and St. Thomas' Dental Institute, London, United Kingdom; S. Gillespie, Department of Medical Microbiology, Royal Free Hospital, London, United Kingdom.

[h] The species identities of group 2a strains of *S. oralis* and *S. mitis* and *S. pneumoniae* 101/87 are those of strain suppliers (4, 15).

RESULTS

Strains included in this study. A complete list of strains examined in this study and their reactions in classical tests used to distinguish pneumococci from oral streptococci is given in Table 1. One aim of this study was to investigate the genetic relationships between the typical capsular and atypical acapsular and/or bile-insoluble pneumococci sometimes encountered in the diagnostic laboratory. As it became clear that some of these isolates were genetically distinct from pneumococci, isolates of the most closely related streptococci (*S. mitis* and *S. oralis*) were included as reference strains. For ease of understanding, the strains in Table 1 have been split into several groups. Group 1 consisted of typical *S. pneumoniae* isolates fulfilling all the conventional criteria of a pneumococcus being serotypable, optochin sensitive, and bile soluble, and the well-characterized laboratory strain R6. Group 2a contained strains of *S. mitis* and *S. oralis* that had been used previously in taxonomic studies (4) included in this study as reference strains. Group 2b consisted of both commensal and pathogenic isolates classified by the suppliers as either *S. mitis* or *S. oralis*. All strains in these latter two groups fulfilled conventional criteria for these organisms in being latex agglutination negative, optochin resistant, and bile insoluble. Group 2c consisted of a group of organisms we have called atypical oral streptococci: this designation is based largely upon the presence of pneumococcal virulence factor genes as described later. However, many of these latter isolates were originally obtained because they proved difficult to classify and were considered unusual; many showed some sensitivity to optochin and displayed aberrant biochemical reactions (data not shown). Group 3 consisted of organisms described as atypical pneumococci largely on the basis of being acapsular, either because they autoagglutinated (rough strains) or because they simply failed to react with any pneumococcal antisera. However, some also showed reduced sensitivity to optochin and some were also bile insoluble. The prototype atypical *S. pneumoniae* strain 101/87, originally described by Diaz et al. (13) on the basis of reactivity with a *lytA* probe (21), was included within this group as a reference strain.

Genetic relationships between isolates. In order to determine the genetic relationships between all of the isolates examined in this study, we examined sequence fragments from three housekeeping genes: *xpt*, *recP*, and *hexB*. By using the neighbor-joining method, dendrograms of genetic relationships between isolates were constructed with sequence data from each of the three genes individually to examine tree topology (data not shown). After this, the sequence data of all three genes were combined and treated as a single contiguous fragment in order to obtain an overall picture of genetic relationships between these isolates, as illustrated in Fig. 1.

Apart from strains characterized as typical pneumococci, the overall picture provided by the data was one of extensive genetic diversity. Only two major groups were strongly supported by bootstrapping. First, many of the *S. pneumoniae* isolates fall into a subgroup, labeled A on the dendrogram, containing all isolates characterized as typical pneumococci—the identical group was also supported by bootstrap values of at least 90% when each of the three genes was considered individually. This group is relatively conserved compared with the remaining organisms: the mean nucleotide diversity between members of this group is 1.42% (range, 0 to 3.0%). A second strongly supported group, labeled group C, contained a much more diverse group of organisms predominantly identified as *S. oralis* and included the *S. oralis* type strain. With the exception of strain AC1372, which harbored a distant *xpt* gene, all of these strains fell within the same group in trees constructed by using the three individual housekeeping genes. A feature of this group was the extensive nucleotide diversity relative to that of isolates of typical *S. pneumoniae*, with a mean diversity of 16.65% (range, 8.7 to 21.2%).

No other major group was strongly supported by bootstrapping with the remainder of the strains forming a diverse group

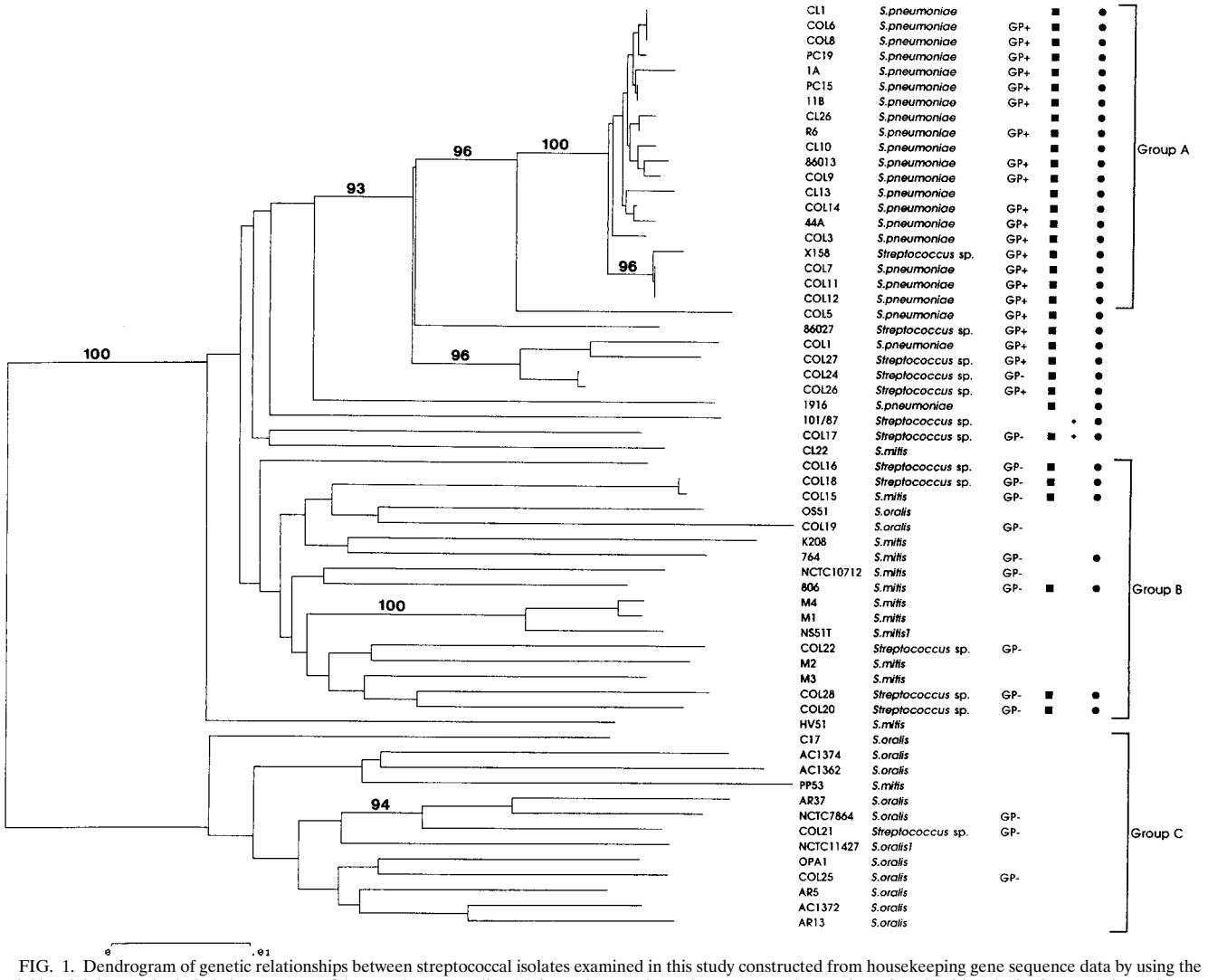

FIG. 1. Dendrogram of genetic relationships between streptococcal isolates examined in this study constructed from housekeeping gene sequence data by using the neighbor-joining method. Only bootstrap confidence values exceeding 90% are shown. The scale represents the number of nucleotide substitutions per site. Where a consistent identification to species level could not be made on the basis of phenotypic criteria isolates have not been given a species designation. A positive or negative result in the Gen-Probe test where performed is indicated by GP+ or GP−. All isolates were screened for the presence of genes encoding *lytA* (■), *lytA101* (◆), and *ply* (●). Groups A, B, and C refer to typical *S. pneumoniae*, *S. mitis*, and *S. oralis* groups, respectively, used to illustrate genetic diversity (see text).

of organisms. Apparently most closely related to, but clearly distinct from, the typical pneumococci are a number of organisms (Col5 to 101/87) classified as atypical pneumococci either on the basis of being acapsular or displaying aberrant reactions to bile and/or optochin. Thus, there appears to be a genuine genetic distinction between typical pneumococci and some of the organisms that have historically been described as atypical pneumococci. The majority of the remaining 20 organisms were identified as *S. mitis*. This loose association of genetically diverse isolates included commensal *S. mitis* isolates, isolates associated with invasive disease, isolates previously characterized in taxonomic studies, and the *S. mitis* type strain. Once again, these organisms were characterized by their extensive genetic diversity. The mean nucleotide diversity within group B (see Fig. 1), which contains most of these organisms, is 16.2% (range, 0.02 to 21.2%).

Genetic relationships of acapsular putative pneumococci. One initial aim of this study was to examine whether acapsular pneumococci are genetically distinct from typical serotypable pneumococci. Of the 16 acapsular organisms included in group 3, nine were closely related to typical capsular pneumococci and presumably represented organisms that either do not express their capsule or else represent as-yet-unrecognized capsular types. However, the remaining organisms (Col5, Col1, 86027, Col27, Col26, 1916, and 101/87) were clearly genetically distinct from typical capsular organisms.

Relationship of phenotype to genetic background. Not one of the classical phenotypic criterion was found to satisfactorily distinguish the genetic groups uncovered in this study. As stated above, many of the acapsular pneumococci are clearly typical pneumococci. Many of the atypical pneumococci and the atypical oral streptococci displayed aberrant reactions to optochin and bile. Recently, use of the GenProbe test has been reported to provide accurate and sensitive identification of pneumococci (13, 14), and we therefore used this test on a subset of our isolates. As might be expected, this test, with a nucleic acid probe to a housekeeping gene, provided the most accurate reflection of genetic relationships determined in this study. In addition to all of the typical pneumococci tested, all of the organisms clustering in the atypical pneumococcal group

TABLE 2. Comparison of extracellular and intracellular hemolytic titers of representative isolates examined in this study

Strain	Strain ID	Hemolytic titer[a]:	
		Cytoplasmic	Extracellular
S. pneumoniae			
Typical	PC19	729	30
	D39	81	30
	D39Δ*ply*	–	–
Atypical	Col27	27	30
S. mitis group			
Typical	NS51^T	–	–
	M3	–	–
Atypical	Col15	243	10
	Col17	729	30
	Col20	729	30
	764	27	10
S. oralis group	NCTC 11427^T	–	–
	Col21	–	–
	Col25	–	–

[a] Hemolytic titer is expressed as the reciprocal of the lowest dilution at which complete lysis of horse blood was seen. Extracellular titers were multiplied by 10 such that they represent a cell number comparable to that used to obtain cytoplasmic titers. –, No hemolytic activity detected.

(with the exception of Col24) as far out as the bile-insoluble strain Col26 gave positive GenProbe reactions.

Distribution of the pneumolysin gene. The distribution of the pnemolysin gene (*ply*) was studied by using PCR primers *ply*up and *ply*dn (Table 1, Fig. 1). The specificity of PCR products was confirmed by probing PCR products transferred to a membrane with the *S. pneumoniae* R6 *ply* gene at high stringency. All isolates falling into the *S. pneumoniae* grouping (both typical and atypical) possessed *ply* and, as expected, isolates within the *S. oralis* grouping did not contain *ply*. Unexpectedly, a number of isolates which fell within the broad group containing previously characterized *S. mitis* isolates were found to possess *ply*. Interestingly, the majority of these isolates whose history is known are disease-associated isolates. In contrast, none of the *S. mitis* isolates previously characterized in taxonomic studies (group 2a) or isolates obtained as commensals or from minor infections of the human oral cavity (group 2b) possessed *ply* (Table 1).

The presence of *ply* in isolates genetically allied to *S. mitis* was confirmed by Southern blot by using the R6 *ply* gene to probe chromosomal DNA (digested with *Pvu*II, which is predicted not to cut within *ply* on the basis of the published sequence) of some of the group 2c organisms (764, Col15, Col17, Col16, Col18, and Col20) and two of the atypical pneumococci, Col26 and Col27 (data not shown). This probe hybridized with all of the chromosomal DNAs and, in agreement with the extensive genetic diversity among these organisms demonstrated by sequence analysis of housekeeping genes, all isolates displayed distinct restriction profiles. An additional interesting finding was that multiple bands in digests from some of these strains hybridized to the *ply* probe. Whether these represent the presence of multiple copies of pneumolysin or merely sequence diversity within a single copy of *ply* requires further investigation.

As final confirmation of the presence of *ply* in nonpneumococcal oral streptococcal isolates, hemolytic titer assays were performed to compare the hemolytic activity associated with some of the atypical *S. mitis* strains with both pneumococci and "conventional" *S. mitis* isolates (Table 2). Both typical pneumococcal controls (strain PC19 and the well-characterized laboratory strain D39) and an atypical pneumococcus (Col27) displayed substantial intracellular and extracellular hemolytic titers. All strains genetically allied to *S. mitis*, but in which *ply* had been detected (Col15, Col17, Col20, and 764), also displayed hemolytic activity in both cytoplasmic and extracellular fractions. The specificity of the assay for pneumolysin was confirmed by the inclusion of a *ply*-deficient D39 mutant strain in these assays. In contrast, *S. mitis* isolates in which *ply* was not detected by genetic approaches, as well as several *S. oralis* isolates, lacked any hemolytic activity under the assay conditions used in this study.

Distribution of the autolysin gene. The distribution of the autolysin gene (*lytA*) was studied by using PCR primers specific for either the typical *lytA* sequence (primers *lytA*up and *lytA*dn) or the atypical *lytA101* sequence (primers *lytA*up and *lytA101*dn) previously reported from the bile insoluble Spanish *S. pneumoniae* isolate 101/87 (Table 1). Once again, specificity was confirmed by using either R6 *lytA* or 101/87 *lytA101* to probe membrane-bound PCR products. As expected, all isolates that form part of the pneumococcal group (typical and atypical) were found to possess *lytA* (Fig. 1). As seen when probing for *ply*, none of the *S. oralis* group isolates were found to possess *lytA*. However, once again, a substantial number of the disease-associated *S. mitis* isolates were found to possess *lytA*. Indeed, all but one (764) of the isolates in which *ply* was detected were also found to possess *lytA*. No isolates were found to be contain *lytA* but lack *ply*. In contrast to *lytA*, a *lytA101*-specific product was detected in only the strain *S. pneumoniae* 101/87, from which this gene was originally isolated (15), and one of the atypical *S. mitis* strains, Col17.

Sequencing of the *lytA* gene found in atypical isolates. It might be expected that isolates possessing *lytA* would display the characteristic bile soluble phenotype associated with this gene (42). This was clearly not the case for some isolates of atypical pneumococci (e.g., Col26 and Col27) and the atypical oral streptococci, Thus, in order to examine the relationship of *lytA* seen in the bile insoluble atypical pneumococci (Col26 and Col27) and some of the atypical oral streptococci (Col15, Col18, Col20, Col16, and Col17) with the known *lytA* sequence (24), PCR products obtained from these strains were sequenced in full. The relationships between these sequences are illustrated in a dendrogram (Fig. 2) comparing the sequences determined here with the original published sequence of *lytA* from strain Rst7, nine *lytA* allelic variants recently reported in typical pneumococci (44), and *lytA101* (101/87). All of the sequences were up to 20% divergent from the published Rst7 *lytA* sequence and other allelic variants seen in typical pneumococci but were much more closely related to *lytA101* sequence (5 to 7% divergent). Interestingly, there was between 0.3 and 4.5% divergence within this group, somewhat higher than the levels of divergence than that reported between allelic variants in typical pneumococci (44).

Relationship of genetic background and clinical association. Many of the *S. oralis* isolates included in this study were blood isolates obtained from immunocompromised patients. However, these isolates involved in infection appeared to be genetically diverse and widely distributed among the isolates characterized in previous taxonomic studies. Similarly, there was no clear separation of the *S. mitis* strains containing *lytA* and *ply* and associated with disease from the commensal isolates of *S. mitis* and those characterized previously in taxonomic studies.

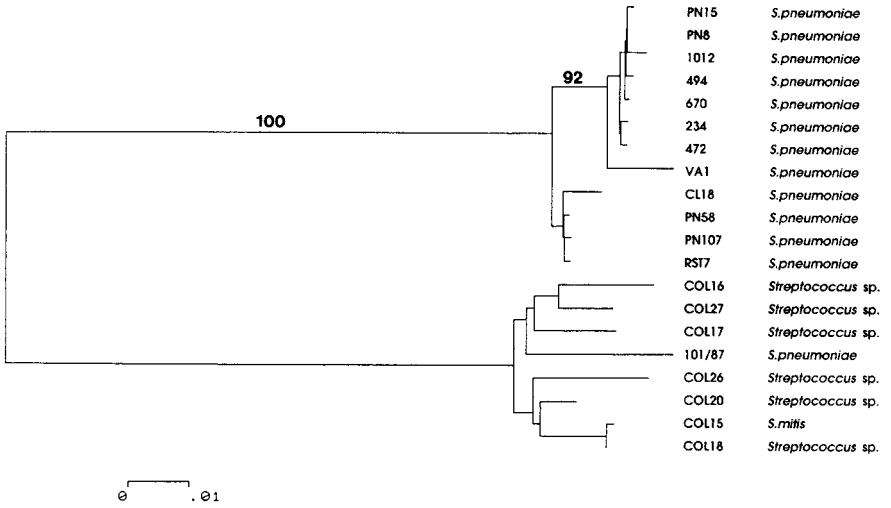

FIG. 2. Dendrogram of genetic relationships between *lytA* sequences examined in this study constructed from gene sequence data by using the neighbor-joining method. Only bootstrap values exceeding 90% are shown. The scale represents the number of nucleotide substitutions per site. The upper group of the tree consists of the previously published *lytA* sequences from strain Rst7, a typical pneumococcal isolate, and a number of allelic variants of *lytA* recently reported from typical pneumococci (44). The lower group contains the sequence *lytA101* from the classical bile-insoluble atypical pneumococcus 101/87 and the *lytA* sequences determined from atypical pneumococci and atypical oral streptococci examined in this study.

DISCUSSION

The primary aim of this study was to examine whether there is a genetic basis to "difficult" or "atypical" isolates sometimes submitted to diagnostic laboratories which have been tentatively identified as pneumococci or oral streptococci but display aberrant phenotypic features. Since the dendrogram of genetic relationships constructed in this study is based on only three housekeeping genes from species that are naturally transformable, we are reluctant to make phylogenetic interpretations based on these data. Recombination is known to occur frequently within pneumococci with the species displaying an epidemic population structure (20, 26, 35). In addition interspecies recombination events between pneumococci and *S. oralis* and *S. mitis* have been seen at least in genes under intense selective pressures, such as penicillin-binding-protein encoding genes (17–19). This possibility of recombination makes phylogenetic interpretation potentially hazardous. Having said this, a number of observations are consistent for all three genes examined when considered individually and in the overall analysis, allowing some confidence in them. Two groupings were strongly supported in all trees. These were a subgroup containing the typical pneumococci and the clear separation of the *S. oralis* group. The atypical organisms (Col5, Col1, Col27, Col24, Col26, 1916, and 86027) were generally placed as the organisms most closely related to pneumococci. In the case of all gene fragments considered individually and the data as a whole the remaining strains (largely identified as *S. mitis*) formed a relatively diverse group of organisms with few consistent branches.

One obvious feature of the data is the extensive diversity seen within the *S. oralis* and *S. mitis* groups when compared to typical pneumococci. This may reflect sampling strategy, although we deliberately selected typical pneumococci thought from previous studies (35) to represent the breadth of genetic diversity within the species. The diversity seen in oral streptococci is consistent with a number of previous reports. For example, among 101 isolates of *S. mitis* examined, 93 distinct *Pvu*II ribotypes were reported (23). Similarly, restriction fragment length polymorphism analysis of *S. mitis* populations in and between individuals found limited sharing of genotypes among family members and some 6 to 13 types in individual subjects (27). Repetitive extragenic palindromic PCR has been used to study *S. oralis* diversity with populations in individuals found to be heterogeneous at a single time point and highly variable when they are monitored longitudinally (3). Recently, PCR-based fingerprinting was also used to examine the relationships between oral streptococci isolated from the blood of neutropenic cancer patients, and all isolates were found to display distinct fingerprint patterns (48).

It is clear from the housekeeping gene sequence data that many isolates that fail to react in the latex agglutination system represent entirely typical pneumococci. It is still not clear why these organisms fail to react with pneumococcal antisera. They may represent as-yet-uncharacterized capsular serotypes, they may possess capsule genes that are nonfunctional or not expressed or, alternatively, they may lack the genes encoding the capsule biosynthetic pathway. We are currently investigating these alternative possibilities using the organisms described in this study. However, a proportion of apparently acapsular isolates did not group with other pneumococci and represent genetically divergent organisms. Thus, there does appear to be a genuine genetic basis to previously described atypical pneumococci which can both fail to react in capsular typing and/or show aberrant biochemical reactions. In light of the extensive genetic diversity within these organisms, it is not surprising that separation on the basis of phenotypic criteria can prove notoriously difficult. The Gen-Probe test was the diagnostic test that appeared to most closely match genetic relationships between isolates with all typical pneumococci and virtually all atypical isolates as far out as Col26 tested giving a positive reaction. Since the basis of this test is hybridization to a housekeeping gene, the correlation with the data presented here is not unexpected. Mundy et al. (36) reported recently that many isolates which show a discordant combination of reactions in capsular typing, optochin sensitivity, and bile solubility tests represent pneumococci on the basis of the Gen-Probe test. In that study the authors suggested the use of Gen-Probe to unequivocally identify isolates showing such discordant pheno-

typic reactions. Our results indicate that many of the isolates identified as pneumococci by Gen-Probe could actually represent organisms which are genetically rather divergent from typical pneumococci.

Perhaps the most surprising finding of this study was the characterization of isolates phenotypically and genetically allied to *S. mitis* harboring genes encoding the putative virulence factors pneumolysin and autolysin normally associated with pneumococci. There has been at least one previous report of a *lytA* probe reacting with optochin-resistant and bile-insoluble streptococci (21), and the use of this gene as a probe to identify atypical pneumococci has been suggested. Our results suggest that using *lytA* probes to identify difficult organisms as atypical pneumococci could also select organisms which are genetically more closely related to *S. mitis*. We are not aware of previous reports of the presence of the pneumolysin encoding gene in oral streptococci. Both *lytA* and *ply* were absent from previously characterized *S. mitis* isolates, as well as commensal isolates obtained in this study. Comparative assays of hemolytic activity associated with *S. mitis* isolates harboring *ply* and lacking *ply* (basing the species identity on observed genetic relationships described in this study) suggested that the hemolysin is actively expressed by the *ply*-containing *S. mitis* strains. In virtually all cases where a strain history was available, the *S. mitis* isolates harboring *lytA* and/or *ply* were associated with respiratory disease. It is thus tempting to speculate that these genes may enhance the pathogenic potential of these organisms relative to the typical commensal organisms. The detection of *ply* and *lytA* in disease-associated putative *S. mitis* strains provided the impetus for us to include *S. oralis* strains associated with invasive disease in immunocompromised patients in this study. However, there was no evidence of the presence of *lytA* or *ply* in members of this group. It is interesting to consider these findings in the light of virulence studies showing that *ply* is important in murine intranasal infection (7, 11) and of a recent signature-tagged mutagenesis study (38) which suggested that *lytA* is important for establishing pneumonia but is not necessary in septicemia. Our data may reflect this in that *lytA*-containing *S. mitis* isolates were associated with pneumonia and respiratory tract disease, while isolates of *S. oralis* from the blood of neutropenic patients did not possess *lytA*. Perhaps the debilitated state of these patients allows ready access of "commensal" isolates to the bloodstream, bypassing the need for any true invasive step. The detection of "pneumococcal" virulence factor genes in *S. mitis* also has implications for attempts to use them as diagnostic targets in PCR (25, 40, 41, 43). Clearly, these organisms could cross-react in such tests, and the community needs to be aware that organisms other than "typical" pneumococci may be identified by such tests although, since they may be pathogenic, detection of these organisms is not necessarily an argument against the development of such tests.

We also performed a preliminary characterisation of the *ply* and *lytA* genes found in *S. mitis* isolates. Interestingly, although PCR products were obtained from these strains by using a *lytA* primer set and, apart from one isolate, not with the *lytA101* primer set, their *lytA* genes were found to be much more closely related to *lytA101* than to the typical *lytA* sequence. The activity of LytA101 is known to be inhibited by sodium deoxycholate, and this property is believed to be responsible for the bile-insensitive phenotype of the host strain 101/87 (15). While the bile insolubility of these strains could reflect their possession of a *lytA101*-like sequence, other possibilities, such as an autolysin refractory cell wall, cannot be ruled out without further experimentation. Probing Southern blots of the same strains with *ply* suggested the possibility of substantial diversity of *ply* within *S. mitis* isolates despite the fact that evidence to date suggests that *ply* in *S. pneumoniae* is a relatively conserved gene (32, 34). An alternative interpretation is that the *S. mitis* isolates may possess multiple copies of this gene. We are currently investigating both of these possibilities. In light of these results it is interesting to speculate on the evolutionary history of this group of organisms. Clearly, the organisms allied to *S. mitis* but harboring *lytA* and *ply* are genetically diverse, as demonstrated by housekeeping gene sequencing, distinct restriction profiles when probing for *ply*, and *lytA* sequencing. This may reflect the fact that these genes are moving freely between organisms with distinct genetic backgrounds. In this respect it is interesting to note all but one strain appeared to harbor both genes and that *ply* and *lytA* are closely linked in the pneumococcal genome lying some 7 kb apart. An alternative, though not mutually exclusive, scenario is that these genes represent ancient characteristics that have been lost by some organisms related to the *S. mitis* group.

We believe it will be important to consider the existence of these atypical organisms in strategies of vaccination against *S. pneumoniae*. These organisms appear to be of pathogenic potential and may be refractory to immune responses generated by immunogens of typical pneumococci. It is also possible that the atypical organisms we have characterized in this study may act as source of DNA in recombination events, generating new alleles of pneumococcal genes under high selective pressure (17–19). In light of this possibility, it is interesting to note that some of the *S. mitis* isolates harboring *lytA* and *ply* (Col15, Col16, and Col18) have been shown to possess competence-stimulating peptides much more closely related to those of pneumococci than to those reported from other isolates of *S. mitis* (45). This raises the possibility that these organisms may show an increased likelihood to donate DNA to pneumococci in horizontal gene transfer events simply because their own competence-stimulating peptides may induce a degree of competence in pneumococci. Although this idea requires experimental confirmation, this could mean that these organisms might serve as a pool of variant DNA which could be readily imported into *S. pneumoniae* genes put under the selective pressure for diversity that inclusion in a potential vaccine might impose. It is thus crucial to examine the nature and extent of genetic diversity in these previously understudied organisms when considering potential vaccine targets in *S. pneumoniae*.

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