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Accuracy of Phenotypic and Genotypic Testing for Identification of *Streptococcus pneumoniae* and Description of *Streptococcus pseudopneumoniae* sp. nov.

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We have identified an unusual group of viridans group streptococci that resemble *Streptococcus pneumoniae*. DNA-DNA homology studies suggested that a subset of these isolates represent a novel species that may be included in the *S. oralis*-*S. mitis* group of viridans group streptococci. We suggest that this novel species be termed *Streptococcus pseudopneumoniae*. A combination of phenotypic and genetic reactions allows its identification. *S. pseudopneumoniae* strains do not have pneumococcal capsules, are resistant to optochin (inhibition zones, less than 14 mm) when they are incubated under an atmosphere of increased CO_2 but are susceptible to optochin (inhibition zones, >14 mm) when they are incubated in ambient atmospheres, are not soluble in bile, and are positive by the GenProbe AccuProbe Pneumococcus test. The bile solubility test is more specific than the optochin test for identification of *S. pneumoniae*. Genetic tests for pneumolysin (*ply*) and manganese-dependent superoxide dismutase (*sodA*) and identification tests with a commercial probe, AccuProbe Pneumococcus, do not discriminate between the new species and *S. pneumoniae*.

Streptococcus pneumoniae is the most common cause of community-acquired pneumonia and is also associated with bacteremia, meningitis, otitis media, and sinusitis, accounting for approximately 3,000 cases of meningitis, 50,000 cases of bacteremia, 500,000 cases of pneumonia, and 7 million cases of otitis media each year in the United States (1). Clinical laboratories must be able to accurately differentiate *S. pneumoniae* from other viridans group streptococci commonly found in clinical samples. An inability to correctly identify *S. pneumoniae* may result in inappropriate antimicrobial therapy.

S. pneumoniae is a member of the *Streptococcus mitis*-*Streptococcus oralis* group (the Smit group) of viridans group streptococci, which includes *S. mitis*, *S. oralis*, *Streptococcus cristatus*, *Streptococcus infantis*, *and Streptococcus peroris*. Differentiation of *S. pneumoniae* from other viridans group streptococci, including members of the Smit group, has conventionally been based on phenotypic characteristics, most commonly by demonstrating optochin (OPT) susceptibility and/or solubility in bile (sodium deoxycholate) (18). Identification by these two tests is satisfactory when isolates from sterile sites are tested (16). However, identification is often problematic when samples from nonsterile sites, such as respiratory samples, are tested. The results of phenotypic testing can result in ambiguous OPT susceptibility and bile solubility (BS) test results (18).

Although they are uncommon, OPT resistance has been reported in *S. pneumoniae* and OPT-resistant subpopulations have also been reported, both of which may lead to problems in identification, especially in association with atypical colony morphologies (4, 13, 36, 42, 45, 46, 55, 60). Gardam and Miller (21) reported that variances in OPT zone sizes are dependent on the test medium and incubation environment used, as some media and environments may result in falsely reduced zone sizes.

Current recommendations for the identification of *S. pneumoniae* suggest incubation in 5% CO_2 for OPT testing, based on reports that up to 8% of pneumococci will not grow aerobically, and that isolates showing zone sizes indicating susceptibility under aerobic incubation conditions are also susceptible when they are incubated in the presence of CO_2 (API 20 Strep identification system for streptococci kit insert, 2003; bioMérieux, Inc., Durham, N.C.). Standardization of OPT susceptibility testing is further confused by the instructions of the Taxo disk manufacturer (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.), which suggest that incubation in CO_2 will enhance growth but reduce zone sizes (BBL Discs for Differentiation of Pneumococci kit insert, 1999; Becton Dickinson Microbiology Systems). The instructions are based on opposing recommendations published in the *Manual of Clinical Microbiology* (49) and a study published in 1971 by Ragsdale and Sanford (48). Ragsdale and Sanford recommended incubation in ambient air, on the basis of the reduced zone sizes obtained when tests were incubated in CO_2, whereas the *Manual of Clinical Microbiology* recommends incubation in CO_2 to enhance the growth of isolates that will not grow aerobically.

Bile-insoluble strains of *S. pneumoniae* have also been reported (39, 43, 44), as have atypical OPT-resistant, BS-negative strains of *S. pneumoniae* (15, 19, 26, 31, 41, 43, 66).

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A review of the literature indicates various methodologies for BS testing (3, 26, 34, 49). BS testing can be performed either with a broth culture of an organism or with colonies growing on a solid medium. Despite methodological variances, BS testing is associated with a sensitivity exceeding 98% and a specificity of 100% (7).

The API 20 Strep system (bioMerieux, Marcy l'Etoile, France) and various commercial biochemical-based tests have been evaluated for their abilities to differentiate viridans group streptococci (20, 24, 28, 32, 33). Generally, these tests do not differentiate *S. pneumoniae* from the Smit group without the addition of OPT and/or BS testing. In well-validated studies, Kikuchi and colleagues (33) and Kawamura (28) reported on a poor correlation between the results obtained with the API 20 Strep and the Rapid ID 32 Strep systems and those obtained by DNA-DNA hybridization within the Smit group by use of the Rapid ID32 Strep kit (28, 33).

A number of commercial rapid test kits are available. These rely on visible detection of an antigen-antibody complex resulting from the reaction between pneumococcal surface antigens and type-specific antibodies (Directigen *S. pneumoniae* test kit insert, 1994 [Becton Dickinson Microbiology Systems, Cockeysville, Md.]; Phadebact Pneumococcus test insert, 2002 [Boule Diagnostics AB, Huddinge, Sweden]; Pneumoslide capsular agglutination test kit insert, 2002 [Becton Dickinson]; Slidex Pneumo-kit insert, 2001 [bioMerieux Canada, Inc., Quebec, Quebec, Canada]). All of these tests are based on the use of Statens Serum Institute Omni serum, a reagent that contains capsular antibodies to all known serotypes of *S. pneumoniae*. Conflicting reports of sensitivity and specificity exist (6, 7, 8, 12, 35, 37, 40, 53, 56, 63). One of the limitations of the agglutination test kits, as with other immunologic techniques, is that pneumococcal cells without capsules may not react, resulting in false-negative results. In addition, antisera may cross-react with some nonpneumococcal viridans group streptococci, resulting in false-positive results (8, 35, 53, 56, 63).

Molecular techniques such as PCR tests for the virulence genes of *S. pneumoniae* (autolysin [*lytA*] and pneumolysin [*ply*]) (9, 10, 15, 23, 26, 29, 30, 31, 39, 40, 50, 52, 59, 61, 62, 65, 66, 67) and penicillin binding protein genes are beginning to gain popularity, as are probe hybridization tests for specific regions of the 16S rRNA gene (16S rDNA) (8, 11, 13, 19, 31, 39, 41, 53, 64, 65).

While some investigators have concluded that amplification of a region of the *lytA* gene can be successfully used to identify *S. pneumoniae* (15, 23, 40, 50), others have demonstrated the presence of *lytA* in members of the Smit group (29, 47, 66) Although it is expected that all virulent isolates of *S. pneumoniae* possess the *lytA* gene, it cannot be presumed that isolates of all other members of the Smit group do not possess the *lytA* gene or may have a facsimile of a *lytA* gene. Therefore, the detection of *lytA* should be used cautiously to identify *S. pneumoniae*.

Detection of the *ply* gene and/or the expression of the Ply protein has also been used in direct detection assays with clinical samples and to differentiate *S. pneumoniae* from other viridans group streptococci, with various rates of success (9, 10, 26, 30, 31, 50, 52, 59, 62, 65, 66, 67). Although DNA amplification methods targeting the *ply* gene initially showed promise as a reliable, efficient method for confirmation of pneumococcal isolates, Kawamura and colleagues (29) and Whatmore and colleagues (66) have reported on the presence of the *ply* gene in strains of *S. mitis* and *S. oralis*, contraindicating its use in differentiating *S. pneumoniae* from other strains of the Smit group (30, 66). Similarly, although latex agglutination methods targeting the protein coded for by the *ply* gene showed promise (9, 31), their use, too, is contraindicated by the finding of the *ply* gene in members of the Smit group other than *S. pneumoniae*.

16S rRNA gene sequencing has limited use for the identification of *S. pneumoniae* because of the genetic similarity between species of the Smit group (22, 27, 29, 43). Whereas DNA-DNA homology studies show 40 to 60% similarity between members of the Smit group, the sequence similarity of the 16S rRNA genes of the type strains of *S. mitis*, *S. oralis*, and *S. pneumoniae* is reportedly greater than 99% (27, 29). Despite these reports of high similarity values for the 16S rRNA gene among members of the Smit group, a DNA probe hybridization test targeting a section of the rRNA specific to *S. pneumoniae* has been developed and is commercially available (AccuProbe *Streptococcus pneumoniae* culture identification test [GP]; Gen-Probe, San Diego, Calif.). Evaluation of the DNA probe hybridization test has resulted in conflicting reports in accuracy (11, 13, 19, 31, 41, 53, 64, 65). Mundy and colleagues (41), Kaijalainen and colleagues (26), and Whatmore and colleagues (66) reported controversial GP results in a study with atypical pneumococci; however, the strains used to evaluate the test were not definitively identified as *S. pneumoniae* (26, 41, 66) A number of studies have used GP to confirm the identities of isolates with discordant results obtained by other methodologies (8, 19, 31, 33, 64). However, there are no published results to support the use of GP as a "gold standard."

Studies with housekeeping genes (i.e., *xpt*, *recP*, *HexB*, and *ddl*) and the manganese-dependent superoxide dismutase gene (*sodA*) have shown promise for the use of these genes to differentiate strains of *S. pneumoniae* from other strains of the Smit group (29, 39, 43, 47, 66).

DNA-DNA reassociation is the gold standard for the identification of strains to the species level and is reportedly the most accurate means of identifying species within the Smit group (28, 29). However, testing of this type is not feasible for most clinical laboratories. Species identification is based on DNA hybridization with the DNA of type strains of greater than 70%. DNA-DNA similarity values based on the entire genome are estimated to be less than 60% for *S. mitis*, *S. oralis*, and *S. pneumoniae* (28).

During the course of study with *S. pneumoniae* clinical isolates we detected a distinct streptococcus. Here we report on the isolation and identification of a streptococcus phenotypically and genetically distinct from *S. pneumoniae* and the other members of the viridans group streptococci, including the Smit group. We propose a species designation for this organism, for which the name *Streptococcus pseudopneumoniae* is suggested.

MATERIALS AND METHODS

Isolates. Thirty-five isolates presumptively identified as *S. pneumoniae* on the basis of susceptibility to OPT comprised the primary set of isolates studied. These 35 strains were a subset of 195 lower respiratory tract isolates identified by the microbiology laboratory at the Queen Elizabeth II Health Sciences Centre (QEII), Halifax, Nova Scotia, Canada. Identification of these isolates, which were subsequently submitted to a national surveillance program, was based on

susceptibility to OPT (Taxo disks; BBL, Becton Dickinson Microbiology Systems), tests for which were conducted on Trypticase soy agar with 5% sheep blood (SBA) incubated in an ambient atmosphere for 18 to 24 h at 35°C, according to the instructions of the manufacturer. The national surveillance program rejected these 35 strains, because in their hands these isolates were OPT resistant and BS negative.

Eighty-seven previously identified BS-positive *S. pneumoniae* isolates were selected from consecutive lower respiratory tract, eye, blood, and cerebrospinal fluid samples and tested by GP and the Phadebact Pneumococcus test, in addition to testing for OPT susceptibility in CO_2 and O_2, to determine the sensitivities of the GP and Phadebact Pneumococcus test methods. A PCR for the presence of the *lytA* and *ply* genes was also performed. Additionally, 35 previously identified viridans group streptococcus species were randomly selected to determine the specificities of the OPT test, BS test, Phadebact Pneumococcus test, GP, and PCR for the presence of the *lytA* and *ply* genes.

A positive control (*S. pneumoniae* ATCC 33400^T) and a negative control (*S. mitis* ATCC 49456^T) were included in all assays. The type strains of all members of the *S. mitis* group described by Facklam were also included in the study for comparison purposes (18).

OPT test. Repeat OPT susceptibility testing was performed in O_2 and 5% CO_2 environments for isolates rejected from the national susceptibility surveillance program. SBA plates were inoculated with colonies from overnight cultures with a loop, the OPT disk was placed in the center of the inoculum, and then the plate was incubated for 18 to 24 h at 35°C in both atmospheres.

BS test. The tube BS test was performed as described in the *Clinical Microbiology Procedures Handbook* (25). Suspensions were prepared in 1 ml of 0.85% NaCl, equivalent to a McFarland 1.0 standard.

GP. GP, based on the rRNA gene sequence, was performed according to the instructions of the manufacturer.

Coagglutination test. Subcultures of isolates were tested for coagglutination by the Phadebact Pneumococcus Test (Boule Diagnostics AB) according to the instructions of the manufacturer.

API 20 Strep system for identification of streptococci. The inoculation, incubation, and reading of the test results were carried out according to the instructions of the manufacturer.

DNA extraction. Template DNA was prepared by suspending a loopful of bacteria, grown overnight on SBA in 200 μl of 0.1 M Tris (pH 7.4). Five microliters of 100 μg of proteinase K per ml in sterile distilled water was added to the suspension, and the mixture was incubated at 37°C for 15 min and then boiled for 10 min. Once the mixture was cooled, samples were centrifuged and 2.5 μl of the supernatant was used as the template in the PCR.

Pneumolysin gene (*ply*) and autolysin gene (*lytA*) PCR. The presence of the *ply* and *lytA* genes in the extracted DNA was determined by PCR with primers specific for a 170-bp region of the *ply* gene and for a 273-bp region of the *lytA* gene, as described previously (61, 67). The PCR products were resolved by electrophoresis on 1.5% agarose gels to which ethidium bromide was added.

***sodA* gene PCR and sequencing.** Forty-one isolates, including the 35 atypical strains, were submitted blindly to the Laboratoire Mixte Pasteur-Necker de Recherche sur les Streptococques et Streptoccies, Faculté de Médecine Necker-Enfants Malades, Paris, France, for *sodA* gene PCR and sequence analysis. Sequencing was carried out as described previously (47).

16S rRNA gene sequence and phylogenetic analysis. DNA extraction and purification were done with a QIAamp DNA Mini kit (Qiagen Inc., Valencia, Calif.), according to the instructions of the manufacturer. Purified genomic DNA was amplified by using the Expand High Fidelity PCR system (Roche, Indianapolis, Ind.) with primers fL1 and rL1 (54). The amplification conditions were 94°C for 5 min; 35 cycles of 94°C for 15 s, 50°C for 15 s, and 72°C for 90 s; and a final single extension of 72°C for 5 min, followed by a hold at 4°C. The products were confirmed by running 10 μl on a 1.0% (wt/vol) agarose gel for 30 min at 75 V. Excess deoxynucleoside triphosphates and primers were inactivated by the ExoSAP-IT method (U.S. Biochemical Corp., Cleveland, Ohio). Cycle sequencing was performed by use of Big Dye (version 3.1) dye terminator chemistry (Applied Biosystems, Foster City, Calif.) by the protocols suggested by Sacchi et al. (51). Excess dyes were removed with magnetic carboxylate beads (Agencourt Bioscience, Beverly, Mass.), and the reaction products were sequenced on an ABI 3100 sequencer (Applied Biosystems). Sequences were assembled by use of the Genetics Computer Group's Seqmerge program (Wisconsin software package, version 10.2) and trimmed to at least two confirming readings. Pairwise comparisons of novel sequences with the sequences in GenBank were performed with the BLAST program. Related entries were aligned in the Pileup program (14) and trimmed to a consensus sequence, and further analysis was performed with Bioedit and Treecon software. With the Bioedit software, the sequences were realigned by use of the Clustal W program (58) with 1,000 bootstraps, and a distance matrix was created. The distances of the aligned sequences were estimated by the Jukes-Cantor method in Treecon software with 1,000 bootstraps, and tree topology was determined by the neighbor-joining method. The final phylogenetic tree was rooted with an outgroup, and bootstrap values above 85% were displayed as percentages.

DNA-DNA reassociation. DNA-DNA reassociation studies were performed with five strains (strains 0207, 0225, 0102, 0103, and 0108) from the BS-negative, OPT-variable, and GP-positive group of isolates. Harvesting and lysis of the bacterial cells were performed as described previously (57). Extraction and purification of DNA and determination of DNA relatedness by the hydroxyapatite hybridization method were performed as described by Brenner and colleagues (5). DNA hybridization experiments were performed at 55°C for optimal DNA reassociation and at the stringent DNA reassociation temperature of 70°C. The levels of divergence within related sequences were determined by assuming that each degree of heteroduplex instability was caused by approximately 1% unpaired bases. Divergence, expressed by the change in melting temperature, is the decrease in the thermal stability (in degrees Celsius) of the heterologous DNA duplex relative to that of the homologous duplexes. Divergence was calculated to the nearest 0.5%. The G+C content was determined by the optical melting temperature and equilibrium buoyant methods by the procedures of Mandel and colleagues (38). All samples were run at least three times, with DNA from *Escherichia coli* K-12 used as a control.

Nucleotide sequence accession numbers. The sequences of strains *S. pseudopneumoniae* ATCC BAA-960^T (CCUG 49455^T) and ATCC BAA-891 (CCUG 48465) and *S. pneumoniae* ATCC 33400^T have been deposited in GenBank under accession numbers AY612844, AY485599, and AY485600, respectively.

RESULTS

Initial isolation and cultural characteristics. One hundred ninety-five nonredundant strains of *S. pneumoniae* were recovered from consecutive lower respiratory tract samples processed in the QEII bacteriology laboratory between 1 November 2000 and 1 November 2002 and submitted to a national susceptibility surveillance program. Isolates were identified as *S. pneumoniae* on the basis of OPT susceptibility in an ambient air atmosphere with zones of inhibition of ≥14 mm.

Subsequently, 46 (21%) of the isolates identified as *S. pneumoniae* were rejected by the surveillance program on the basis of OPT resistance (zone size, <14 mm) when they were incubated in CO_2 and negative BS test results. Upon retesting of these 46 isolates, 8 strains were found to be OPT and BS positive and were serotyped at the Centers for Disease Control and Prevention (CDC); 2 strains were OPT, BS, and GP negative and were presumed to be viridans group streptococci. One strain was omitted because we could not repeat the original reactions. The remaining 35 strains are the subject of the remainder of this study and are called OPT-variant (OPT-V) strains.

Of the 87 previously identified BS-positive pneumococcal isolates that were selected from consecutive lower respiratory tract, eye, blood, and cerebrospinal fluid samples for further testing, only one produced an zone of inhibition of <14 mm with an OPT disk in an atmosphere of increased CO_2; and all isolates demonstrated zones of inhibition ≥14 mm with OPT disks when the test was incubated in ambient air. All strains were positive by the tube BS test, including the one strain with an inhibition zone <14 mm with the OPT disk. All of the typical *S. pneumoniae* strains were Phadebact test and GP positive. All 87 previously identified *S. pneumoniae* strains demonstrated amplification products of the appropriate sizes for the *ply* and *lytA* genes (Table 1).

The OPT-V strains are so called because they are, for the most part, OPT test positive when they are incubated in am-

TABLE 1. Phenotypic and genotypic test results for typical BS *S. pneumoniae*, OPT-V strains, and viridans group streptococci

Isolate	No. of strains											
	OPT susceptible						Bile soluble	Phadebact test positive	GP positive	Autolyin gene PCR		Pneumolysin gene PCR positive
	In CO_2 atmosphere with zone size of:			In O_2 atmosphere with zone size of:						~200 bp	~270 bp	
	6 mm	8–13 mm	≥14 mm	6 mm	8–13 mm	≥14 mm						
S. pneumoniae (n = 87)	2	1	84	0	0	87	87	87	87	0	87	87
OPT-V (n = 35)	20	13	2	0	5	30	0	35	27/33	24	0	35
Viridans group streptococci (n = 35)	33	1	1	30	2	3		10	1	2	1	4
S. milleri (n = 6)	6	0	0	6	0	0	0	0	0	0	0	0
S. mitis (n = 16)	14	1	1	11	2	3	0	8	1	2	1	4
S. oralis (n = 6)	6	0	0	6	0	0	0	1	0	0	0	0
S. salivarius (n = 6)	6	0	0	6	0	0	0	1	0	0	0	0
S. sanguis (n = 1)	1	0	0	1	0	0	0	0	0	0	0	0

bient air atmosphere but OPT test negative when they are incubated in an atmosphere of increased CO_2. Under an atmosphere of increased CO_2, 20 of the 35 strains produced no inhibition zone with OPT disks, 13 produced small inhibition zones (8 to 13 mm), and 2 had zone sizes larger than 14 mm. When the OPT disk test was incubated under ambient air atmosphere, 5 strains produced reduced zones (8 to 13 mm) and 30 produced zones >30 mm (Tables 1 and 2).

After 24 h of incubation on SBA plates in a CO_2 atmosphere, the OPT-V strains grew as small and sometimes tiny or pinpoint, shiny, smooth, grayish alpha-hemolytic colonies that resembled *S. pneumoniae* colonies only slightly more than they resembled colonies of viridans group streptococci. Although some of the variant streptococci resembled viridans group streptococci, others looked more like *S. pneumoniae*.

None of the 35 OPT-V strains were positive by the tube BS test. All 35 OPT-V isolates agglutinated in the Phadebact Pneumococcus test (Table 1). Twenty-seven of the 33 strains tested by GP were positive, resulting in the identification of *S. pneumoniae*. With the exception of strain 0211, which tested positive by GP at CDC, the remaining five OPT-V, BS-, and GP-negative strains (strains 0107, 0203, 0206, 0209, and 0218) were not investigated further.

Twenty-four of the 35 OPT-V strains demonstrated a truncated amplification product of approximately 200 bp by PCR with primers targeting a 273-bp region of the *lytA* gene. Eleven isolates failed to produce a product by PCR with the *lytA* gene primers (Tables 1 and 2). All 35 of the OPT-V isolates demonstrated the expected 170-bp *ply* gene amplification product (Tables 1 and 2).

Only 1 of the 35 viridans group streptococci had a zone of inhibition >14 mm in tests with OPT disks when the strains were incubated in an atmosphere with increased levels of CO_2. However, when the strains were incubated in ambient air, three viridans group streptococcal strains had inhibition zones >14 mm. The strains with small zones of inhibition and with zone sizes ≥14 mm were all identified as *S. mitis*. None of the 35 strains of viridans group streptococci were tube BS test positive. Ten of the 35 viridans group streptococci tested coagglutinated by the Phadebact test; 9 of these were previously identified as *S. mitis*, while 1 was identified as *S. oralis* and 1 was identified as *S. salivarius* (Table 1). Two of these strains demonstrated an amplification product of the appropriate size for the *ply* gene, and one demonstrated a truncated *lytA* gene product (Tables 1 and 2). Four of the 35 viridans group streptococci, all identified as *S. mitis*, tested positive by the PCR for *ply*.

Comparison of OPT, BS, and GP (QEII and CDC). The results obtained by the OPT disk test with incubation under an atmosphere with increased CO_2 or in ambient air are listed in Table 2. The inhibition zones seen with incubation in ambient air were much larger than those seen with incubation in an atmosphere of increased CO_2. When the OPT disk test was incubated under an atmosphere with increased CO_2 levels, no zones of inhibition were observed with 18 of the 35 strains in either laboratory. Nine additional strains had no inhibition zones when they were tested in the CDC laboratory, but they had small inhibition zones (9 to 11 mm) when they were tested in the QEII laboratory. Four strains had larger inhibition zones in the QEII laboratory, while three others had larger inhibition zones in the CDC laboratory. Only two strains in each laboratory had inhibition zones >14 mm and would have been erroneously identified as *S. pneumoniae* if no other test had been used for identification. When the OPT disk test was incubated in an ambient air atmosphere, 29 strains in each laboratory showed inhibition zones ≥14 mm. Only two strains showed zones of inhibition less than 14 mm in both laboratories. There was no pattern of increased inhibition zone sizes in one laboratory versus the other; inhibition zones were larger in the QEII laboratory for 14 strains, and inhibition zones were larger in the CDC laboratory for 18 strains; inhibition zone sizes were identical for 3 strains at both laboratories. The tube BS test was used in both the CDC and the QEII laboratories. None of the 35 OPT-V strains were positive in either laboratory (Table 2). The GP results were positive and correlated for 27 of the 28 strains tested in both laboratories. One strain, 0211 (Table 2), was positive at the CDC laboratory but negative at the QEII laboratory.

Serotyping. All 35 OPT-V strains listed in Table 1 failed to react with CDC pneumococcal typing antiserum. All 11 strains of *S. pneumoniae* tested were positive by tests with the CDC Quellung typing antisera: 3 strains were type 6B; 2 strains were

TABLE 2. Phenotypic and genotypic test results for OPT-V strains of streptococci[a]

QEII isolate identifier	CDC isolate identifier	No. of strains positive for TaxP in[b]:				Bile soluble		GP result		Autolysin	Pneumolysin	*sodA* sequencing result	DNA reassociation result
		CO_2		O_2									
		QEII	CDC	QEII	CDC	QEII	CDC	QEII	CDC				
0105	294-03	0	0	9	14	−	−	+	+	T	+	Spn	ND
0106	295-03	0	0	25	17	−	−	+	+	T	+	Spn	ND
0108	297-03	0	0	23	18	−	−	+	+	−	+	Spn	Spsp
0110	299-03	0	0	26	25	−	−	+	+	−	+	ND	ND
0202	248-03	0	0	23	34	−	−	+	+	T	+	Spn	ND
0207	253-03	0	0	16	30	−	−	+	+	−	+	Spn	Spsp
0210	256-03	0	0	16	17	−	−	+	+	−	+	Spn	ND
0213	259-03	0	0	12	14	−	−	+	+	T	+	Spn	ND
0216	262-03	0	0	15	10	−	−	+	+	T	+	Spn	ND
0219	265-03	0	0	17	21	−	−	+	+	T	+	Spn	ND
0220	266-03	0	0	14	16	−	−	+	+	T	+	Spn	ND
0214	260-03	0	0	20	20	−	−	+	+	T	+	Spn	ND
0225	276-03	0	0	20	15	−	−	+	+	T	+	ND	Spsp
0228	280-03	0	0	30	10	−	−	+	+	T	+	ND	ND
0234	286-03	0	0	19	12	−	−	ND	+	T	+	ND	ND
0211	257-03	0	0	23	33	−	−	−	+	T	+	Spn	ND
0203	249-03	6	0	10	10	−	−	−	−	−	+	Spn	ND
0101	287-03	11	0	18	17	−	−	+	+	−	+	Spn	ND
0102	288-03	9	0	13	16	−	−	+	+	T	+	Spn	Spsp
0103	290-03	9	0	19	17	−	−	+	+	−	+	Spn	Spsp
0109	298-03	10	0	20	28	−	−	+	+	T	+	Spn	ND
0111	300-03	9	0	17	19	−	−	+	+	T	+	Spn	ND
0208	254-03	10	0	13	10	−	−	+	+	T	+	Spn	ND
0212	258-03	10	0	17	19	−	−	+	+	T	+	Spn	ND
0215	261-03	11	0	19	18	−	−	+	+	T	+	Spn	ND
0232	284-03	10	0	20	15	−	−	+	+	T	+	ND	ND
0201	247-03	9	8	26	35	−	−	+	+	T	+	Spn	ND
0233	285-03	12	10	19	15	−	−	ND	+	−	+	ND	ND
0204	250-03	0	9	16	24	−	−	+	+	−	+	Spn	ND
0205	251-03	0	8	24	25	−	−	+	+	T	+	Spn	ND
0206	252-03	13	15	24	24	−	−	−	−	T	+	Spn	ND
0209	255-03	19	17	29	21	−	−	−	−	−	+	Spn	ND
0222	269-03	11	7	19	22	−	−	+	+	T	+	*S. mitis*	ND
0107	296-03	0	0	12	20	−	−	−	−	T	−	*S. mitis*	ND
0218	264-03	15	11	19	20	−	−	−	−	−	+	*S. mitis*	*S. mitis*

[a] Abbreviations: T, truncated amplification product (approximately 200 bp); expected product size, 270 bp; Spn, *S. pneumoniae*; Spsp, *S. pseudopneumoniae*; ND, not done.

[b] Obtained by using 6-mm Taxo Differentiation Discs for Pneumococci (BBL, Becton Dickinson Microbiology Systems) containing 5 μg of ethylhydrocupreine hydrochloride.

type 14; 2 strains were type 31; and 1 strain each was type 7F, 11A, 16F, and 18C. Four viridans group streptococci did not react with any of the pooled antisera from CDC.

Evaluation of the API 20 Strep identification system for streptococci. Biochemical testing was done with 13 isolates; 10 OPT-V strains (1 in duplicate), 1 OPT-V but GP-negative strain, and 1 *S. pneumoniae* reference strain. This system failed to definitively identify any of the isolates to the species level (Table 3). In all cases, additional testing was suggested; often, additional testing involved OPT susceptibility testing and/or solubility in bile, two tests that in most cases would have been performed prior to use of the API test strip. Two identical API profiles (0060000 and 0060400) were observed for four strains each, and three other strains had unique profiles. In some cases the additional tests did not result in a definitive identification, which was the correct identification, since four of these strains (strains 0102, 0103, 0108, and 0207) have been shown to be a new species of the viridans group streptococci.

***sodA* gene sequencing.** Thirty-six isolates, including viridans group streptococci, OPT-V strains, and typical *S. pneumoniae* strains, were submitted blindly to the Laboratoire Mixte Pasteur-Necker de Recherche sur les Streptococques et Streptoccies, Faculté de Médecine Necker-Enfants Malades, for *sodA* gene PCR and sequence analysis (47). Seven typical *S. pneumoniae* strains based on conventional phenotypic and serologic testing (two type 6B strains; two type 18C strains; and one strain each of types 7F, 14, and 31) demonstrated *sodA* sequences identical to that of the *S. pneumoniae* type strain (ATCC 33400); these are listed as 217, 221, 226, 223, 224, 216, and 104, respectively, in Fig. 1. Twelve OPT-V strains (strains 108, 105, 212, 201, 204, 220, 102, 202, 109, 214, 106, and 208 in Fig. 1) clearly belong to the pneumococcal lineage. Two viridans group streptococci (strains 206 and 209, both of which were OPT positive and BS and GP negative) also joined this *S. pneumoniae* group. The *sodA* sequences of eight other OPT-V strains (strains 101, 103, 111, 207, 210, 211, 213, and 219 in Fig.

TABLE 3. Biochemical test results (API 20 Strep system) for subset of strains

Isolate	API profile	Identification	Additional tests[a]						
			Salacin	NaCl	Levane	OPT	NO_2-N_2	Glycerol	Dextran
0101, OPT-V	4060401	73.5% *S. mitis* I	+ (−)	−		R			
		15.8% *S. oralis*	−	NT	−	NT			
		Possible *S. pneumoniae*				S			
0102 (1), OPT-V	0060000	69.9% *Gemella morbillorum*					−[b]		
		28.5% *Gemella haemolysans*					+		
0102 (2), OPT-V	0062400	94.6% *S. mitis*	+ (−)			R		−	
		Possible *S. pneumoniae*	−			S		+ (−)	
0103, OPT-V	0060000	69.9% *G. morbillorum*					−[b]		
		28.5% *G. haemolysans*					+		
0105, Opt-V	0060400	81.1% *S. mitis*	+ (−)			R		−	
		Possible *S. pneumoniae*	−			S		+ (−)	
0106, OPT-V	0060400	81.1% *S. mitis*	+ (−)			R		−	
		Possible *S. pneumoniae*	−			S		+ (−)	
0108, OPT-V	0060400	81.1% *S. mitis* I	+ (−)			R		−	
		Possible *S. pneumoniae*	−			S		+ (−)	
0109, OPT-V	0060000	69.9% *G. morbillorum*					−[b]		
		28.5% *G. haemolysans*					+		
0110, OPT-V	0060000	69.9% *G. morbillorum*					−[b]		
		28.5% *G. haemolysans*					+		
0111, OPT-V	0060400	81.1% *S. mitis* I	+ (−)			R		−	
		Possible *S. pneumoniae*	−			S		+ (−)	
0207, OPT-V	4062400	85% *S. mitis*	+ (−)			R		−	
		Possible *S. pneumoniae*	−			S		+ (−)	
0107, GP negative	0040401	85.6% *S. mitis*	+ (−)			R		−	
		Possible *S. pneumoniae*	−			S		+ (−)	
Reference strain *S. pneumoniae*[c]	0240450	49.2% *S. sanguis*	+	V		R			+
		20.0% *S. oralis*	−	100%		NT			78%
		17.5% *S. pneumoniae*	−	NT		−			−
		9.0% *S. mitis*	+ (−)	−		R			−

[a] Percentages are percent positive values. Abbreviations: NO_2-N_2, nitrate to nitrite; R, resistant; S, susceptible; + (−), usually positive, though some may be negative; V, variable; NT, not tested.
[b] Favors anerobic growth.
[c] The reference strain was BS negative and OPT susceptible by the disk method.

1) differed from that of the *S. pneumoniae* type strain by 1 bp. One viridans group streptococcus (strain 0225) also joined this group. Three OPT-V strains (strains 0215, 0222, and 0205) and three viridans group streptococci (strains 0107, 0218, and 0203) were more closely aligned with *S. mitis* than with *S. pneumoniae* according to their *sodA* sequences (Fig. 1).

16S rRNA gene sequencing. Strain 0108 (CDC SS-1575) was chosen as the type strain and was deposited in the American Type Culture Collection (ATCC BAA-960^T) and in the Culture Collection of the University of Goteborg (CCUG 49455^T). The relationship of the type strains of 12 species of viridans group streptococci, including the *S. mitis*, *S. oralis*, and *Streptococcus sanguinis* groups and *S. pneumoniae*, with the *Streptococcus pyogenes* type strain representing the genus is shown in Fig. 2. Pairwise comparisons of the 16S rDNA sequences (a total of 1,468 bp) showed that *S. pseudopneumoniae* is very closely related to *S. pneumoniae*, with a difference of only 5 bp between the two species, which corresponds to 99.7% identity.

DNA reassociation. Labeled DNA of the type strain representing *S. pseudopneumoniae* (strain 0108) was used in DNA-DNA reassociation experiments to estimate the reassociation values for the pneumococcal type strain, ATCC 33400, and strains 0102, 0103, 0207, 0225, and 0218 (Table 4). Strains, 0102, 0103, 0207, and 0225 formed a cluster with the reference strain of *S. pseudopneumoniae*. All of these strains were OPT-V, BS negative, and GP positive. The reassociation values were greater than 70%, and the divergence was less than 3% for these strains. These values are within the limits for species identity and can be used to indicate that all five strains are a single species. The reassociation values for the type strain of *S. pneumoniae* were below the limits for it to be included in the species. The values for strain 0218 were also below the limits required for it to join the *S. pseudopneumoniae* group. This strain was GP negative; it was included in the analysis to show that one phenotypic characteristic is reason enough to not include it in this new species.

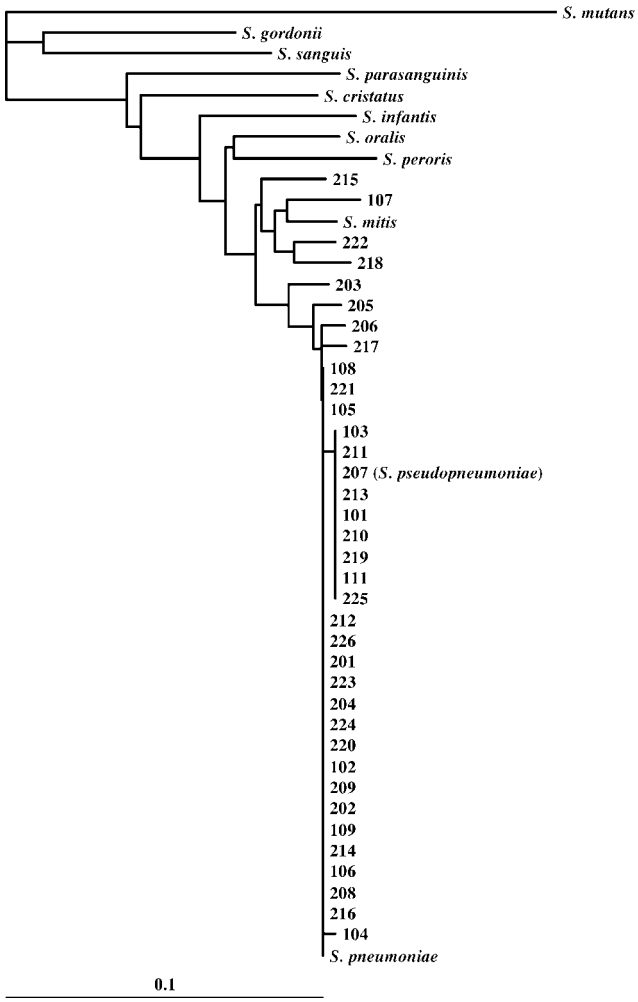

FIG. 1. Phylogenetic tree, based on comparative analysis of the *sodA* gene sequences, showing the relationship of *S. pseudopneumoniae* sp. nov. and related species of the genus *Streptococcus*.

DNA-DNA reassociation values for the reference strain of *S. pseudopneumoniae* were also compared with those for all known viridans group streptococci in the *S. mitis*, *S. oralis*, and *S. sanguinis* groups. For each of the representatives of these species, the reassociation values with the reference strain were well below 70%, indicating that strain ATCC BAA-960^T is distinct and separate from all of the other viridans group streptococcal species listed in Table 4. The G+C content of strain ATCC BAA-960^T was 39.5 mol%. That value is similar to the published values for streptococci (49). Thirty-five of the OPT-V strains (Table 1) displayed the same phenotypic and genotypic characteristics as reference strain *S. pseudopneumoniae* 0108^T (ATCC-BAA-960^T).

DISCUSSION

During the process of evaluating a number of pneumococcal identification tests, we encountered a group of strains that were OPT-V, BS negative, and GP positive that shared phenotypic characteristics with viridans group streptococci and pneumococcal strains. Subsequent experiments showed that these strains belong in the Smit group. We have designated this new species *S. pseudopneumoniae*. *S. pseudopneumoniae*, which most closely resembles pneumococci when it is grown on SBA in CO_2, grew as smooth, slightly mucoid, grayish, alpha-hemolytic colonies that resembled *S. pneumoniae* in appearance. The appearance of these strains may vary depending on the base medium, incubation conditions, and age of the colonies. Strains were susceptible to OPT when they were incubated in an ambient environment by the disk assay, but they were resistant (no inhibition zone) or indeterminate (inhibition zone, 8 to 13 mm) when they were incubated in CO_2. We are not aware of any previous studies that have characterized these strains; however, there is similarity between the isolates identified as *S. pseudopneumoniae* in this study and atypical pneumococcal strains previously identified and described in the literature (15, 43, 66). None of these strains reacted with pneumococcal typing antiserum, indicating a lack of pneumococcal polysaccharide antigens.

OPT susceptibility testing is the test most commonly used in clinical microbiology laboratories to screen alpha-hemolytic colonies for the identification of pneumococci; however, it remains unclear whether incubation in CO_2, as recommended in the *Manual of Clinical Microbiology* (49), or ambient atmosphere, as recommended by the manufacturer of Taxo OPT test disks, is optimal for the identification of pneumococci. The data collected in this study suggest that while incubation in CO_2 on SBA is more specific, it is less sensitive than BS testing. Three of 87 BS-positive *S. pneumoniae* isolates would have been missed by OPT susceptibility testing in CO_2 alone (3.4%). Two of these strains had no zone at all when they were incubated in CO_2 but had zones of 18 and 22 mm, respectively, when they were incubated in O_2. The other strain, which had a small inhibition zone of 12 mm, would have been tested for BS, and a correct identification would have resulted. Although OPT susceptibility testing in O_2 alone selected these isolates, it also selected five isolates (14%) previously identified as *S. mitis*. Approximately 18% of the isolates from lower respiratory tract samples, identified as *S. pneumoniae* by OPT susceptibility testing in O_2, fit into this variant group of viridans group streptococci. Further studies are needed to determine whether this new species of viridans group streptococci, *S. pseudopneumoniae*, has pathogenic potential. Until the pathogenicity of *S. pseudopneumoniae* is determined, our recommendation for OPT susceptibility testing on SBA is incubation in CO_2, unless BS testing is also performed simultaneously. If *S. pseudopneumoniae* is subsequently found to be pathogenic, OPT susceptibility testing of BS-negative, GP-positive isolates in both CO_2 and O_2 environments may be useful for the differentiation of *S. pneumoniae* from *S. pseudopneumoniae*.

During the course of the study, we observed variations in the results of BS tests performed with the same isolates by technologists at three different institutions using different methodologies. Microbiology texts are not consistent on the method that should be used to test for BS. The *Manual of Clinical Microbiology* (49) suggests one method in the chapter on streptococci, but it refers to a different method in the section on reagents, stains, and media. The method described in the *Manual of Clinical Microbiology* appears to be more consistent in identifying strains with weak expressions of solubility in bile (data not shown).

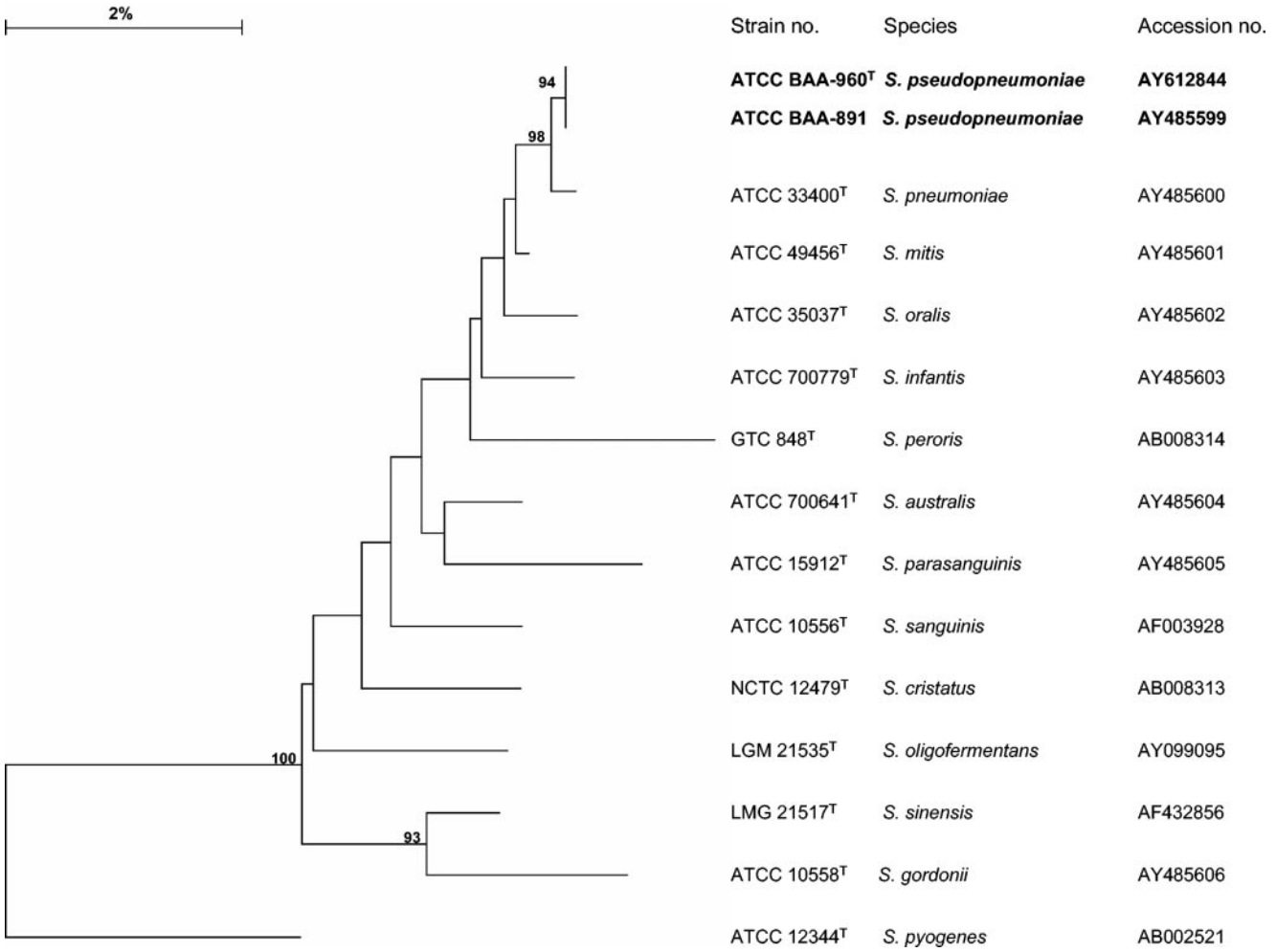

FIG. 2. Phylogenetic tree, based on comparative analysis of the 16S rDNA sequences, showing the relationship of *S. pseudopneumoniae* sp. nov. and related species of the Smit group. The limit criterion used to show the bootstrap numbers was above 85%, and *S. pyogenes* was used as the outgroup.

Identification of autolysin and/or pneumolysin virulence genes initially showed promise for use in the identification of pneumococci (9, 10, 15, 23, 26, 29, 30, 31, 39, 40, 50, 52, 59, 61, 62, 65, 66, 67). However, although these genes are present in all pneumococcal strains, a number of investigators have also reported their presence in viridans group streptococci other than *S. pneumoniae* (28, 29, 47, 66). In this study, we were able to demonstrate PCR products of the expected size for the pneumolysin virulence gene (*ply*) in 4 of the 16 *S. mitis* strains tested and in all 35 of the OPT-V strains subsequently identified as *S. pseudopneumoniae*. An amplification product of the expected size for the *lytA* gene (~273 bp) was also found for 1 of the 16 *S. mitis* strains, and a truncated product of approximately 200 bp was demonstrated for 2 of the 16 *S. mitis* strains and 22 of the OPT-V strains subsequently identified as *S. pseudopneumoniae*. It is unclear whether the *S. pseudopneumoniae* strains that tested negative for the autolysin gene are truly negative or whether they harbor remnants of the autolysin gene that were not amplified by our primers. Sequencing of the regions upstream and downstream of the autolysin gene is necessary to determine this.

Not surprisingly, the API 20 Strep system (bioMerieux)

TABLE 4. DNA-DNA hybridization reactions of OPT-V, bile-insoluble, and GP-positive strains[a]

Strain	RBR[b] at 55°C	*D*[c]	RBR at 70°C
ATCC BAA-960T	100		
S. pneumoniae ATCC 33400T	55	3.5	28
0102	82	0.5	50
0103	81	1.5	75
0207	84	2.0	74
0225	83	1.0	77
0218[d]	53	4.5	42
S. mitis ATCC 49456T	43		
S. oralis ATCC 35037T	34		
S. infantis ATCC 700779T	18		
S. australis ATCC 700641T	17		
S. parasanguinis ATCC 15912T	18		
S. cristatus ATCC 51100T	18		
S. sanguinis ATCC 10556T	12		
S. peroris ATCC 700780T	25		
S. gordonii ATCC 10558T	14		
S. oligofermentans LMG 21535T	28		

[a] The reference strain was *S. pseudopneumoniae* ATCC BAA-960T.
[b] RBR, relative binding ratio.
[c] *D*, divergence, calculated to the nearest 0.5%.
[d] GP negative.

showed poor specificity with the isolates tested, and in all cases, additional testing was required before an identification could be made. Although only a small number of isolates tested were in this study, the results are consistent with those published previously (20, 28, 33). Kikuchi and colleagues (33) and Kawamura (28) reported a poor correlation between the results obtained with the API 20 Strep and Rapid ID 32 Strep systems and those obtained by DNA-DNA hybridization within the Smit group by use of the Rapid ID32 Strep kit (28, 33). Fordymacki and colleagues (20) also reported a poor correlation of the results obtained by using the API 20 Strep, Rapid ID 32 Strep (bioMerieux), and Streptoplast PPL 18 (HTL, Warsaw, Poland) systems with *S. pneumoniae* and recommended that these systems not be used for the identification of pneumococci.

Although the sensitivity of the Phadebact Pneumococcus coagglutination kit was excellent (87 of 87 *S. pneumoniae* isolates were correctly identified), its specificity was extremely poor. All 35 of the variant isolates, subsequently identified as a new species of streptococci, *S. pseudopneumoniae*, were positive; and 10 of the 35 viridans group streptococci tested also gave positive results: 8 of 16 strains were *S. mitis*, 1 of 6 strains was *S. oralis*, and 1 of 6 strains was *S. salivarius*. On the basis of these results, the Phadebact Pneumococcus coagglutination kit is not recommended for use for the identification of *S. pneumoniae* in the absence of a confirmatory test, such as the BS test.

16S rRNA gene sequencing showed a very close relationship between *S. pneumoniae* and *S. pseudopneumonaie*. Emler and colleagues (S. Emler, N. Liassine, J. Pawlowsky, B. Hirschel, P. Rohner, and R. Auckenthaler, Abstr. 97th Gen. Meet. Am. Soc. Microbiol., poster D-155, 1997) demonstrated a high degree of homology and minor differences in distinct positions that allowed differentiation of closely related species, such as *S. mitis* and *S. pneumoniae*. However, other investigators have found that although sequencing of 16S rDNA is an excellent tool for the identification of most organisms, it does not have sufficient discriminatory power to differentiate members of the mitis group of streptococci (22, 27, 29, 43).

The GP investigators have chosen a sequence of the rRNA gene for their test that does not discriminate between *S. pneumoniae* and *S. pseudopneumoniae*. Our results indicate that the portion of the sequence may be identical for both species. OPT-resistant, BS-negative, and GP-positive isolates have been observed in other laboratories; however, DNA homology studies were not performed to definitively identify these isolates (15, 19, 26, 31, 41, 43, 66; W. H. Haas [University of Washington], personal communication, 2003). It seems likely, on the basis of test characteristics, that these isolates may, in fact, be *S. pseudopneumoniae* and not *S. pneumoniae*, as reported. Further studies with definitive identification methods, such as DNA homology analysis, and an assortment of BS-negative, OPT-resistant, and OPT-V strains are required to determine the specificity of GP. Although it is possible that the manufacturer has targeted a region of the 16S rRNA gene that distinguishes *S. pneumoniae* from other viridans group streptococci, preliminary results from this study suggest that GP lacks the discriminatory power necessary to separate *S. pneumoniae* from *S. pseudopneumoniae*.

Sequencing of an internal segment of the *sodA* gene also showed promise for the identification of *S. pneumoniae*; however, the results obtained in this study indicate that sequencing of the region targeted by our primers lacks the discriminatory power to differentiate *S. pseudopneumoniae* from *S. pneumoniae*. This may indicate that the new species, *S. pseudopneumoniae*, has a *sodA* gene identical to that of *S. pneumoniae*.

Difficulties with the identification of *S. pneumoniae* and other naturally competent organisms may be caused by horizontal transfer of heterologous genes and evolution, resulting in an evolutionary soup. Taxonomists have a tendency to create static divisions of microorganisms, but in reality, nature contains many mosaic organisms that do not clearly fit into any particular species over time.

The accurate identification of viridans group streptococci, including *S. pneumoniae*, remains problematic. Until such time as the pathogenicity of *S. pseudopneumoniae* is determined, pneumococcal identification protocols must include tests that can differentiate this new species from its closely related, clinically significant neighbor, *S. pneumoniae*. Observations made during the course of this study suggest that BS testing is the most accurate of the phenotypic tests used in the clinical laboratory for the identification of pneumococci, although problems may be encountered when one is dealing with weakly bile-soluble strains. OPT susceptibility testing remains a useful screening test; however, the manufacturer's recommendation for incubation in an ambient environment should be changed to a recommendation for incubation in CO_2 to exclude selection of *S. pseudopneumoniae*.

Sequencing of the autolysin genes of *S. pneumoniae* and *S. pseudopneumoniae* may provide regions that are useful for the development of a probe that will differentiate *S. pneumoniae* from *S. pseudopneumoniae* yet that will be feasible for routine use in the clinical laboratory, as GP is. Further investigation is required.

DNA homology testing of isolates previously described in the literature as atypical pneumococci is of particular interest to us and may provide evidence that isolates are more closely related to *S. pseudopneumoniae* than to *S. pneumoniae* or another member of the Smit group.

Further studies are required to determine the pathogenic potential of *S. pseudopneumoniae*. The identification of 35 of 195 isolates (18%) from lower respiratory tract specimens in this study would seem to indicate that it may have pathogenic potential, but without additional information this potential cannot be validated at this time.

There was a subgroup of strains other than *S. pseudopneumoniae* or *S. pneumoniae* that likely represent viridans group streptococci. These isolates exhibited the same OPT susceptibility variabilities demonstrated by *S. pseudopneumoniae*, but they were GP negative. DNA-DNA homology studies were not performed with these isolates.

Conclusions. DNA-DNA homology studies suggest that a subset of our isolates represent a novel species of the Smit group that is distinct from *S. pneumoniae* and *S. mitis*.

Performance of OPT susceptibility testing in a 5% CO_2 environment is necessary to reduce the possibility of misidentification of *S. pneumoniae*. Solubility in bile appears to be the most reliable phenotypic test for the differentiation of *S. pneumoniae* from other species in the Smit group, including a novel species, designated *S. pseudopneumoniae*. *S. pseudopneumo-*

niae can be differentiated from *S. pneumoniae* and *S. mitis* by the demonstration of insolubility in bile, a zone size for resistance or indeterminate susceptibility (<14 mm with a 6-mm OPT disk) when it is incubated in CO_2 and a zone size for susceptibility (≥14 mm with a 6-mm OPT disk) following incubation in an ambient environment, and a positive reaction by GP. Standardization of OPT susceptibility testing and BS testing is required for consistent, reproducible results.

S. pseudopneumoniae strains react positively by genetic probe tests for the pneumolysin gene (*ply*), the manganese-dependent superoxide dismutase gene (*sodA*), and the rRNA gene by a commercial assay, GP. It is also probable that this species has a modified autolytic gene (*lytA*).

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