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Streptococcus pseudopneumoniae Identification by Pherotype: a Method To Assist Understanding of a Potentially Emerging or Overlooked Pathogen

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The recent identification of *Streptococcus pseudopneumoniae* (pseudopneumococcus) has complicated classification schemes within members of the "mitis" streptococcal group. Accurate differentiation of this species is necessary for understanding its disease potential and identification in clinical settings. This work described the use of the competence-stimulatory peptide ComC sequence for identification of *S. pseudopneumoniae*. ComC sequences from clinical sources were determined for 17 strains of *S. pseudopneumoniae*, *Streptococcus pneumoniae*, and *Streptococcus oralis*. An additional 58 ComC sequences from a range of sources were included to understand the diversity and suitability of this protein as a diagnostic marker for species identification. We identified three pherotypes for this species, delineated CSP6.1 (10/14, 79%), CSP6.3 (3/14, 21%), and SK674 (1/14, 7%). Pseudopneumococcal ComC sequences formed a discrete cluster within those of other oral streptococci. This suggests that the *comC* sequence could be used to identify *S. pseudopneumoniae*, thus simplifying the study of the pathogenic potential of this organism. To avoid confusion between pneumococcal and pseudopneumococcal pherotypes, we have renamed the competence pherotype CSP6.1, formerly reported as an "atypical" pneumococcus, CSPps1 to reflect its occurrence in *S. pseudopneumoniae*.

The mitis group of streptococci includes nasopharyngeal colonizers such as *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus pneumoniae*, and the recently classified *Streptococcus pseudopneumoniae* (2). Of these, *S. pneumoniae* (pneumococcus) is responsible for more than a million deaths annually and is responsible for diseases such as otitis media, pneumonia, septicemia, and meningitis. However, invasive diseases caused by other related viridans group streptococci had been documented (16, 26, 31).

Some strains of *S. pseudopneumoniae*, along with *S. mitis* and *S. oralis*, have often been classified previously as "atypical pneumococci," because of their similarity to *S. pneumoniae*. These organisms share ≥99% identity in 16S rRNA gene sequences (2, 21, 43). Optochin sensitivity and bile solubility, the two standard pneumococcal phenotypic identification tests, have proven to be inconclusive for differentiating pneumococci from these atypical strains (3, 4, 9, 10, 17, 19, 20, 22, 27, 29, 32, 34, 38, 39, 49). Virulence factors that were once thought to be exclusive to the pneumococcus, such as pneumolysin (encoded by *ply*) and autolysin A (encoded by *lytA*), have been detected in commensal streptococcal species (18, 35, 49), compromising their specificity as species identification markers. The pathogenic potential of *S. pseudopneumoniae* (the pseudopneumococcus) has been demonstrated in a murine model (12) as well as in humans (2, 18, 23, 24, 28, 40). Rapid, correct identification of this organism in the clinical setting is essential for diagnosis and for understanding its disease potential. A simple, unequivocal method to identify *S. pseudopneumoniae* would be valuable.

Streptococci are competent for genetic transformation. In the case of *S. pneumoniae*, this is mediated by the competence-stimulatory peptide (CSP) encoded by the *comC* gene (13). CSP sequences differ between species and within species; different versions within species are known as pherotypes (48). We report the distribution of the *comC* sequence in strains of *S. pseudopneumoniae* and show that it may prove a valuable method to identify the organism rapidly.

MATERIALS AND METHODS

Clinical specimens and bacteria. A total of 17 clinical specimens of *S. pseudopneumoniae*, *S. pneumoniae*, and *S. oralis* were collected at the Royal Free Hospital Microbiology Laboratory between the years 1993 and 2010 (Table 1). Sixteen samples were from patients with lower respiratory tract (LRT) infections, and a single strain was isolated from a normally sterile site. Samples were plated on Columbia blood agar (Oxoid, Cambridgeshire, United Kingdom) in 5% CO_2 at 35°C in an attempt to cultivate bacteria, and colonies suggestive of pneumococci based on morphology and alpha-hemolysis were tested for optochin sensitivity.

Genomic DNA extraction for amplification. Genomic DNA from LRT samples was extracted by a modified Chelex method as described previously (47). The supernatant containing the DNA was used as the amplification template. For culture-positive clinical specimens, genomic

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TABLE 1 Streptococcal strains included for this study

Species	Strain	Clinical isolation site	Accession no.[c]	Source or reference
S. pseudopneumoniae	N452	Blood	Not deposited	This study
	RFH504	LRT[a]	Not deposited	This study
	RFH543	LRT	Not deposited	This study
	RFH686	LRT	Not deposited	This study
	RFH687	LRT	Not deposited	This study
	RFH827	LRT	Not deposited	This study
	RFH905	LRT	Not deposited	This study
	RFH999	LRT	Not deposited	This study
	874	Unknown[b]	AJ240773	48
	ATCC BAA-960	LRT	Not deposited	This study
	IS7493	LRT	YP004769537	40
	PT5479	Naso/oropharynx	Not deposited	41
	PT5779	Naso/oropharynx	Not deposited	41
	SK674	Unknown	Not deposited	25
S. pneumoniae	RFH324	LRT	Not deposited	This study
	RFH410	LRT	Not deposited	This study
	RFH577	LRT	Not deposited	This study
	RFH815	LRT	Not deposited	This study
	RFH864	LRT	Not deposited	This study
	RFH904	LRT	Not deposited	This study
	VA1	Unknown	AJ240789	48
	41G	Unknown	AJ240766	48
	CSP2.1b	Nasopharynx	Not deposited	46
	Pn24	Unknown	AJ240759	48
	Pn59	Unknown	AJ240793	48
	Pn13	Unknown	AJ240792	48
	101/87	Unknown	AJ240791	48
	SK676	Unknown	Not deposited	25
S. mitis	Col15	Unknown	AJ240762	48
	Col16	Unknown	AJ240763	48
	NCTC 10712	LRT	AJ240795	48
	NCTC 12261	Naso/oropharynx	AJ000875	15
	B5	Unknown	AJ000871	15
	B6	Unknown	AJ000865	15
	Hu8	Unknown	AJ000866	15
	SK137	Unknown	Not deposited	25
	SK145	Unknown	Not deposited	25
	SK262	Unknown	Not deposited	25
	SK272	Unknown	Not deposited	25
	SK564	Unknown	Not deposited	25
	SK596	Unknown	Not deposited	25
	SK598	Unknown	Not deposited	25
	SK599	Unknown	Not deposited	25
	SK601	Unknown	Not deposited	25
	SK602	Unknown	Not deposited	25
	SK608	Unknown	Not deposited	25
	SK609	Unknown	Not deposited	25
	SK611	Unknown	Not deposited	25
	SK612	Unknown	Not deposited	25
	SK614	Unknown	Not deposited	25
	SK615	Unknown	Not deposited	25
	SK667	Unknown	Not deposited	25
	SK675	Unknown	Not deposited	25
S. oralis	RFH623	LRT	Not deposited	This study
	RFH831	LRT	Not deposited	This study
	Col19	Unknown	AJ240794	48
	NCTC 11427	Naso/oropharynx	AJ000873	15
	DSM 20066	Unknown	AJ000874	15

(Continued on following page)

TABLE 1 (Continued)

Species	Strain	Clinical isolation site	Accession no.[c]	Source or reference
	SK153	Unknown	Not deposited	25
	SK305	Unknown	Not deposited	25
	SK34	Unknown	Not deposited	25
	SK39	Unknown	Not deposited	25
	SK571	Unknown	Not deposited	25
	SK597	Unknown	Not deposited	25
	SK610	Unknown	Not deposited	25
	SK92	Unknown	Not deposited	25
S. gordonii	NCTC 3165	Gum	AJ000870	15
	NCTC 7865	Endocardium	X98110	14
	NCTC 7868	Unknown	X98109	14
S. infantis	SK140	Unknown	Not deposited	25
	SK282	Unknown	Not deposited	25
	SK283	Unknown	Not deposited	25
	SK350	Unknown	Not deposited	25
S. cristatus	NCTC 12479	Unknown	AJ000876	15
S. peroris	ATCC 700780	Tooth	EFX39822	NCBI genome

[a] LRT, lower respiratory tract.
[b] Unknown, isolation site not specified in previous publications or not available on ATCC or NCTC database.
[c] Accession numbers are absent where strain sequences were under 200 bp and thus not deposited. Nucleotide sequences for pherotypes associated with strains characterized in this study are given in Table S2 in the supplemental material (pherotype CSPps1a is associated with *S. pseudopneumoniae* strains N452, RFH504, RFH543, RFH687, RFH905, and RFH999; pherotype CSPps2b is associated with *S. pseudopneumoniae* strains RFH686, RFH827, and ATCC BAA-960; pherotype CSP1c is associated with *S. pneumoniae* strains RFH324, RFH410, RFH577, RFH815, RFH864, and RFH904; and pherotype CSP6.2d is associated with *S. oralis* strains RFH623 and RFH831). Undeposited sequences for other strains listed are available in the indicated reference.

DNA was extracted using the Wizard genomic DNA purification kit (Promega) or by the heat lysis method (30).

Presumptive identification of *S. pseudopneumoniae*. Quantitative PCR (qPCR) using primers and probes specific for Spn9802 (1, 44) and *lytA* (5) was performed sequentially to differentiate between *S. pneumoniae* (both positive) and *S. pseudopneumoniae* (Spn9802 positive, *lytA* negative). To monitor PCR inhibition, a SPUD potato gene internal amplification control (IAC) was included in each reaction using primers targeting *phyB* of *Solanum tuberosum* (36). For *lytA* and Spn9802 qPCR assays, amplification reactions using 25-μl mixtures containing 1× Platinum quantitative PCR SuperMix-UDG (Invitrogen), a final concentration of 7 mM $MgCl_2$, primers and probes, 4×10^{-7} μM IAC template DNA (Sigma-Aldrich), and 5 μl of template DNA (see Table S1 in the supplemental material) were performed. Negative and positive controls were performed for each qPCR assay. Amplification was performed using a Rotor-Gene Q (Qiagen) with the following conditions: an initial hold cycle at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. The PCR data were acquired at the end of each cycle and analyzed by the instrument software (Qiagen). Samples with cycle threshold (C_T) values ≤35 for the *lytA* or Spn9802 target were considered positive; samples that had no C_T value for either the *lytA* or Spn9802 target but that did have a C_T value ≤40 for the IAC target were considered negative, and samples with a no C_T value for either the *lytA* or Spn9802 target and no C_T value for the IAC target were considered inhibited. Samples positive for both *lytA* and Spn9802 were considered to be *S. pneumoniae* positives, and samples positive for Spn9802 and negative for *lytA* were considered to be presumptively *S. pseudopneumoniae* positive. All reactions were performed in triplicate.

Amplification of *comC*. Forward and reverse primers were designed to bind *comC* at positions 592 to 611 and 873 to 892, respectively (accession number U33315). The primer sequences and primer reaction concentrations are indicated in Table S1 in the supplemental material. For strains grown on agar, genomic DNA was extracted from bacterial cells from a fresh overnight culture on Columbia blood agar by the heat lysis method as described previously (30). In addition to primers, each reaction mixture contained 2 μl of template DNA, 0.3 μl *Taq* polymerase (5 U/μl) (Invitrogen), 1× PCR buffer (10×), 3 μM $MgCl_2$, and 0.6 mM deoxynucleoside triphosphates (dNTPs; Promega), made up to a final volume of 50 μl with DNase/RNase-free distilled water (Gibco).

MLST. The primers used for multilocus sequence typing (MLST) have been described previously (8). MLST was performed with the same reaction components and concentrations indicated for the *comC* amplification described above.

Amplicon purification and sequencing of *comC* and MLST loci. Amplicons were analyzed with 1.5% (wt/vol) agarose gel electrophoresis. Amplicons with the expected band sizes were purified using a PCR purification kit (Qiagen) according to the manufacturer's instructions. Cycle sequencing and sequence analysis were performed in the same manner for both PCRs (see below). Purified DNA was sequenced using BigDye Sequencing Terminator v.3.1 (Applied Biosystems), and sequences were analyzed on the 3130 genetic analyzer (Applied Biosystems) and viewed on Bionumerics software (version 5).

Publicly accessible sequences of streptococcal CSP. Additional streptococcal ComC amino acid sequences were included in this study for the construction of a phylogenetic tree (see below). Sequences obtained from previous publications, with accession numbers, are indicated in Table 1. Sequences for which no accession numbers are listed have not been deposited in GenBank. The sequences can be found in the sources given in Table 1.

Construction of a *comC* phylogenetic tree. Multiple alignment of *comC* sequences was constructed with ClustalW functionality on MEGA version 5.05 (45). For both analyses, neighbor-joining trees were constructed based on alignment data. Bootstrap support of 1,000 repetitions was performed.

RESULTS

***S. pseudopneumoniae* CSP sequences.** It was possible to identify ComC sequences for nine pseudopneumococcal strains where

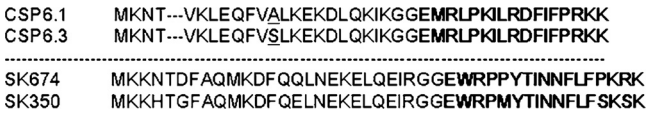

FIG 1 Pherotypes of *S. pseudopneumoniae* CSP6.1, CSP6.3, and SK674. The mature region of the peptide is in boldface after the double glycine. CSP6.1 and CSP6.3 differ by a single amino acid at position 12 (alanine in CSP6.1 and serine in CSP6.3; underlined). SK674 is a presumptive pseudopneumococcus (25), with an extended ComC compared to CSP6.1 and CSP6.3, and shares higher identity to *S. infantis* SK350. A total of three pseudopneumococcal pherotypes were characterized.

CSP sequences were derived directly from samples submitted to our laboratory from patients with LRT and invasive infections. These samples were presumed to contain pseudopneumococci based on either Spn9802-positive and *lytA*-negative qPCRs (eight strains), bile insolubility and optochin-variable phenotypic traits (one strain, N452), or MLST (one strain, N452). The pherotype of the control strain, ATCC BAA-960, was also characterized in this study. Two pherotypes were detected in these nine strains, six (N452, RFH504, RFH543, RFH687, RFH905, RFH999) of which were associated with CSP6.1. To avoid confusion with pherotypes of other oral streptococci, we propose that CSP6.1 be named CSPps1, where "ps" represents the pseudopneumococcus. The three remaining strains, BAA-960, RFH686, and RFH827, had a ComC sequence that has not been reported before; this sequence is identical to that of CSPps1 in size, differing by an alanine-to-serine substitution at position 12 of the propeptide. We propose that this pherotype be classified as CSPps2 (Fig. 1).

CSP sequences of four strains (IS7493, PT5479, PT5779, and SK674) were available from reports in earlier publications (25, 41, 48). All but that of SK674 were identical to CSPps1. Thus, more than 70% of the presumptive *S. pseudopneumoniae* strains in this study were associated with CSPps1 (Table 2). SK674 has an extended ComC of 54 amino acids, most similar to a pherotype characterized in *Streptococcus infantis* SK350, with six amino acid substitutions, three of which are in the mature peptide region (25) (Fig. 1 and Table 2).

Phylogenetic analysis of streptococcal pherotypes. A phylogenetic tree constructed from alignment of streptococcal pherotypes shows that, by pherotype, streptococci fall into two major groups (Fig. 2). *S. pneumoniae*, *S. pseudopneumoniae*, *S. mitis*, and some *S. oralis* strains belong to one group (group 1), while a more divergent and loosely defined group (group 2) consists of predominantly *Streptococcus gordonii*, *S. infantis*, *Streptococcus peroris*, *Streptococcus cristatus*, and most of the remaining *S. oralis* strains. SK674, formerly classified as a pseudopneumococcus (25), clustered near members of *S. infantis* by ComC alignment. All of the remaining *S. pseudopneumoniae* pherotypes are grouped in a separate cluster in close relation to other species, notably *S. oralis* and *S. mitis* (Fig. 2).

DISCUSSION

We have characterized pherotypes associated with *S. pseudopneumoniae* by comparing strains available to us, amplifying sequences collected from lower respiratory tract samples and collecting publicly available ComC sequences for this organism and related streptococcal species. We have shown that CSP6.1 (or CSPps1) is the commonest pherotype among *S. pseudopneumoniae* strains found in different geographical regions. Pherotype CSP6.1 was previously considered to be a rare pherotype of an "atypical nontypeable pneumococcal" strain, 874, based on multilocus sequence analysis (33, 48). Its classification as a pneumococcus may stem from its possession of *ply* and *lytA*, which were once considered suitable genetic markers for this organism (33). However, it is known that these two genes are not specific to *S. pneumoniae* (18, 35, 49), and we have been unable to find a report of CSP6.1 being found in a strain unequivocally identified as *S. pneumoniae*. Based on these observations, we hypothesize that strain 874 is a strain of *S. pseudopneumoniae*. Pneumococcal strain 101/87, associated with CSP5, was described as an "atypical pneumococcus" and could not be serotyped by Whatmore et al. (48), and this strain is most likely to be *S. pseudopneumoniae*. Phylogenetic analysis of CSP sequences in this study suggests that CSP5 is most closely related to *S. mitis* or *S. oralis* pherotypes that cluster together. Thus, we believe that CSP6.1 is associated with *S. pseudopneumoniae* and that, to differentiate pseudopneumococcal pherotypes from ComC sequences from other organisms, they should be designated CSPps1 instead of CSP6.1.

In this study we have identified a new pherotype associated with *S. pseudopneumoniae* and have designated this CSPps2. *S. pseudopneumoniae* pherotypes form a distinct cluster within those of other oral streptococcal species, suggesting that pseudopneumococcal pherotypes could be species specific and could be used as a simple diagnostic tool.

In contrast, strain SK674, a *S. pseudopneumoniae* strain identified based on clustering of housekeeping gene sequences (25), clustered closely in ComC sequences of *S. infantis* strains. One might argue that SK674 acquired a divergent *comC* from *S. infantis* by horizontal gene transfer. While interspecies transfer of the competence operon has been documented (15), the lack of such an event in our collection of over 200 pneumococcal strains indicates that this is relatively rare (our unpublished data). SK674 has a genome size of 1.87 Mbp (25), comparable to those of other *S. infantis* strains (1.74 to 1.88 Mbp) and much smaller than the genome size of the pseudopneumococcal strain IS7493 (2.1 Mbp) (40). It seems that the alternative explanation, that SK674 is actually a strain of *S. infantis*, is more likely. Additional analysis of SK674, such as DNA-DNA hybridization and *lytA* sequence analysis, may shed light as to the true identity of this strain.

From the recently characterized genome sequence of *S. pseudopneumoniae* strain IS7493, it was concluded that CSP-mediated induction of fratricide does not take place based on the absence of *comC* in this strain (40). We were, however, able to locate the gene that is identical to that of CSPps1. Thus, we can conclude that this *S. pseudopneumoniae* strain does contain the necessary gene sequences for production of a competence peptide and that a homologous gene is found in all pseudopneumococcal strains analyzed thus far, strengthening the use of this gene as a diagnostic marker.

TABLE 2 Distribution of pseudopneumococcal pherotypes

Pherotype	No. of strains (% of 14)	Strain(s)
CSP 6.1 (CSPps1)	10 (71)	N452, RFH504, RFH543, RFH687, RFH905, RFH999, IS7493, 874, PT5479, PT5779
CSPps2	3 (21)	BAA-960, RFH686, RFH827
SK674	1 (7)	SK674

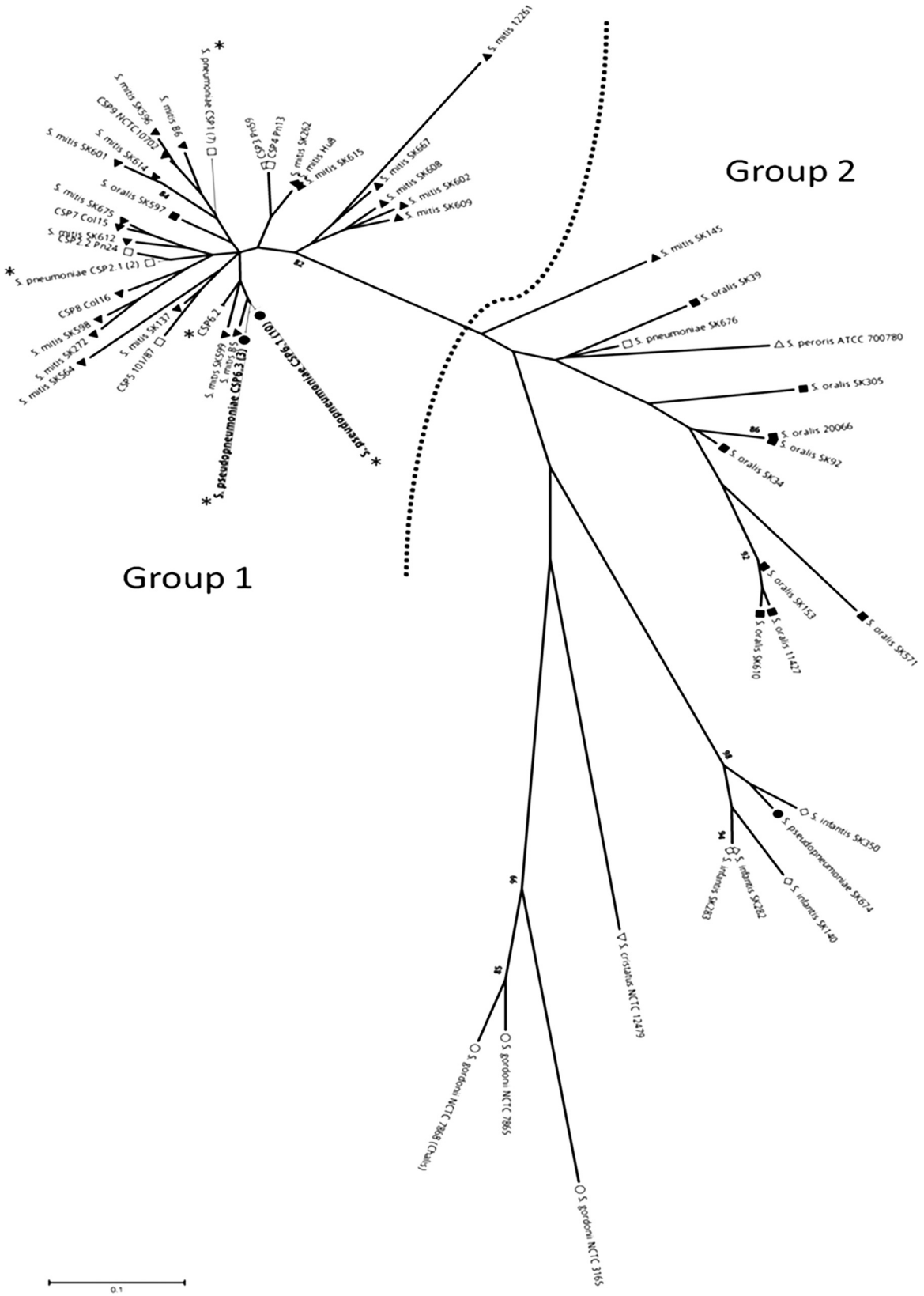

FIG 2 Neighbor-joining phylogenetic tree of ComC amino acid sequences of streptococcal species of the mitis group. Two clusters of ComC based on amino acid sequence similarities (groups 1 and 2) are separated by a dotted line. Each pherotype is labeled according to the classified species: filled circle, *S. pseudopneumoniae*; open square, *S. pneumoniae*; filled triangle, *S. mitis*; filled square, *S. oralis*; open circle, *S. gordonii*; open upright triangle, *S. peroris*; open diamond, *S. infantis*, open inverted triangle, *S. cristatus.* Asterisks indicate multiple strains of the same pherotype that have been compressed for clarity. Numbers of strains with the same pherotype are indicated in parentheses. Pseudopneumococcal strains of these groups are indicated in Table 2. The pneumococcal CSP1 pherotype includes strains VA1, RFH324, RFH410, RFH577, RFH815, RFH864, and RFH904. The pneumococcal CSP2.1 pherotype includes strains 41G and CSP2.1b. CSP6.2 pherotypes include SK671 (*S. mitis*), Col19 (*S. oralis*), RFH623 (*S. oralis*), and RFH831 (*S. oralis*). The phylogenetic tree was built with 1,000 bootstrap repetitions, with support over 80 indicated. The ruler indicates amino acid substitutions per site. The tree was constructed with MEGA 5.05.

S. pseudopneumoniae is usually identified as acapsulate, bile insoluble, and intermediately optochin resistant in 5% CO_2 and optochin susceptible in ambient O_2 (2). These tests can be difficult to standardize in the laboratory. Identification by *comC* sequencing would allow a rapid method of definitive diagnosis as the competence ligand gene is conserved across streptococcal species and the pseudopneumococcal *comC* sequences appear to provide taxonomic information, similar to the case of *gyr* in *Serratia* species (6). Previously reported genetic approaches to differentiate *S. pseudopneumoniae* from *S. pneumoniae* relied on targeting *lytA*, *cpsA*, *aliB*-like ORF2, *ply*, *psaA*, Spn9802, and *sodA* (2, 5, 7, 16, 24, 37, 42, 44); however, these are inconclusive. While multilocus sequence analysis can reveal divergence between species by their housekeeping and virulence gene fragments (11, 25), these methods are too cumbersome for large-scale and routine clinical diagnosis. Recent accounts of detection of pseudopneumococcus in carriage and symptomatic hosts with antibiotic resistance necessitate its accurate diagnosis as an emerging causative agent of disease (23). A recent report suggested that sequencing *recA* could differentiate between *S. pneumoniae* and *S. pseudopneumoniae* (43), but the study was performed with a smaller number of pseudopneumococcal strains solely from North America. Here we propose that pherotyping may be a promising diagnostic alternative based on the clustering of pseudopneumococcal pherotypes from different continents.

In conclusion, we propose that CSP sequence analysis can provide rapid accurate differentiation of *S. pseudopneumoniae* from closely related species *S. pneumoniae*, *S. mitis*, and *S. oralis*. With this in mind, we anticipate that some strains currently classified as atypical pneumococci can be identified as pseudopneumococci based on ComC sequencing. Use of ComC sequencing will simplify the gathering of data to understand the disease potential of this organism, which may be now emerging as a pathogen. To add to this observation, we would encourage other laboratories to sequence "atypical pneumococcal" strains to provide more sequences to confirm whether ComC may be used as a rapid marker for the identification of this emerging pathogen.

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