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Highly Penicillin-Resistant Multidrug-Resistant Pneumococcus-Like Strains Colonizing Children in Oeiras, Portugal: Genomic Characteristics and Implications for Surveillance[▽]†

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While performing surveillance studies in Oeiras, Portugal, designed to describe the impact of pneumococcal conjugate vaccine on colonization, we observed an increase from 0.7% in 2003 to 5% in 2006 in the prevalence of penicillin resistance (MIC of 2 to 6 mg/liter) among presumptively identified pneumococcal isolates. Although 15 of the 22 penicillin-resistant isolates obtained in 2006 were optochin resistant, they were bile soluble and thus considered to be bona fide pneumococci. This study aimed to clarify the nature of these isolates by using a combination of phenotypic and genotypic approaches that included routine strategies for pneumococcal identification, multilocus sequence analysis (MLSA), and comparative genomic hybridization (CGH). By MLSA, all isolates were classified as "streptococci of the mitis group" that, however, were distinct from typical *Streptococcus pneumoniae* or *Streptococcus mitis*. A single isolate was identified as *Streptococcus pseudopneumoniae*. CGH confirmed these findings and further indicated that a considerable part of the proposed pneumococcal core genome is conserved in these isolates, including several pneumococcal virulence genes (e.g., *pavA*, *spxB*, *cbpE*, and *cbpD*). These results suggest that among pneumococci and closely related streptococci, universal unique phenotypic and genetic properties that could aid species identification are virtually impossible to define. In pneumococcal colonization studies, when atypical strains are found, MLSA and CGH are informative tools that can be used to complement routine tests. In our study, after correct identification of the penicillin-resistant true pneumococci, we found that penicillin resistance levels among pneumococci remained stable from 2003 to 2006.

Streptococcus pneumoniae is a bacterial pathogen that frequently colonizes the nasopharynx of humans, particularly young children of preschool age. Colonization is mostly asymptomatic and only rarely results in disease (3). However, when disease does occur, it may range from a mild infection such as otitis media to severe septicemia or meningitis. Globally, the morbidity and mortality associated with pneumococcal infections are extremely high. A recent report from the WHO estimated that 0.7 to 1.0 million deaths occur annually among children <5 years of age as a result of pneumococcal infections (50).

Four phenotypic characteristics are classically used in the diagnostic laboratory for the presumptive identification of *S. pneumoniae*: colony morphology (colonies with a depression in the center showing alpha-hemolysis on sheep blood agar), optochin susceptibility, deoxycholate (DOC) solubility (commonly referred to as bile solubility), and a positive reaction with antipneumococcal polysaccharide capsule antibodies (20). In particular, optochin susceptibility and deoxycholate solubility have been associated with high sensitivity and specificity (between 98% and 100%). However, a number of studies have reported on sporadic optochin-resistant *S. pneumoniae* isolates (28, 34) and rare deoxycholate-insoluble strains (32).

On the other hand, some nonpneumococcal oral streptococci (such as *Streptococcus mitis*) may have a colony morphology similar to that of pneumococci but are classically optochin resistant, bile insoluble, and do not react with antipneumococcal polysaccharide capsule antibodies (25). Still, optochin-susceptible nonpneumococcal isolates have been described occasionally (20), as well as isolates with positive cross-reactions with antipneumococcal polysaccharide capsule antibodies (11). Recently, a new species—*Streptococcus pseudopneumoniae*—was described (1). *S. pseudopneumoniae* isolates were found to be resistant to optochin when incubated in an atmosphere enriched in CO_2 but were optochin susceptible when incubated in ambient atmosphere. As a result, *S. pneumoniae* isolates may be difficult to distinguish from closely related species such as *S. pseudopneumoniae* and *S. mitis*.

Since the biochemical tests commonly used are not always sufficient to distinguish *S. pneumoniae* from other closely related upper respiratory streptococci, molecular approaches based on amplification of ubiquitous pneumococcal genes,

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such as pneumolysin (*ply*) and autolysin (*lytA*), have been proposed (27). However, homologues of *lytA* and *ply* genes have been detected in strains of closely related streptococcal species (15, 29, 37, 49). Recently, detection of *piaA* (which encodes a lipoprotein component of two iron ABC transporters) has been proposed as a diagnostic tool for pneumococci as it was suggested to be specific for this species (47). Some authors also described 16S rRNA and *sodA* as good targets for identification of *S. pneumoniae* although *sodA* does not distinguish *S. pneumoniae* from *S. pseudopneumoniae* (1, 12, 21). The construction of phylogenetic trees from the concatenated sequences of the genes used for multilocus sequence typing (MLST)—an approach commonly termed multilocus sequence analysis (MLSA)—has also been proposed as a good alternative molecular technique to differentiate pneumococci from other closely related streptococci (9, 18).

In recent years, we have been studying atypical pneumococci recovered from colonization samples collected from children attending day care centers (DCCs) in Portugal. We first described a collection of over 200 nonserotypeable pneumococci which were mostly multidrug resistant and displayed low-level resistance to penicillin. All isolates were found to be true pneumococci that lacked a capsular operon. The isolates were genetically diverse although close to half belonged to a single lineage (39). Overall, these isolates were relatively abundant in asymptomatic carriers. The second group of atypical pneumococci that we described consisted of isolates resistant to optochin. Again, all strains were found to be true pneumococci and genetically diverse, and, in this case, most expressed a pneumococcal capsular type (30).

In the current study, we describe a third set of presumptively identified atypical pneumococcal strains. These isolates were all obtained in 2006 during a cross-sectional study designed to describe the impact of the seven-valent pneumococcal conjugate vaccine in colonization. Strikingly, 5% (22 of 441) of the presumptively identified pneumococcal isolates were found to have penicillin MICs ranging from 2 to 6 mg/liter, a value that between 2001 and 2003 never exceeded 1.7% and was 0.7% in 2003. Of note, 15 of these isolates, although resistant to optochin, were bile soluble and, thus, according to routinely accepted criteria, were considered to be bona fide pneumococci. Since such high MICs of penicillin are extremely rare among this population, we initiated a detailed characterization of these isolates consisting of classical strategies for pneumococcal identification, MLSA, and comparative genomic hybridization (CGH). Ten other optochin-resistant, bile-soluble isolates with penicillin MICs ranging from 0.064 to 0.75 mg/liter were also identified and further characterized.

We report here that these isolates are not true pneumococci but have phenotypic properties and genomic determinants that are frequently associated with *S. pneumoniae*, challenging their correct identification by several currently accepted assays.

MATERIALS AND METHODS

Strain collection. Strains used in this study were isolated during January and February of 2006 from the nasopharynx of preschool children attending DCCs in Oeiras, Portugal. Swabs were inoculated in tryptic soy agar (TSA) containing 5% defibrinated sheep blood supplemented with 5 mg/liter gentamicin and incubated overnight in anaerobic jars at 37°C to select and identify *S. pneumoniae*. As controls, we used *S. pneumoniae* strains R6 and TIGR4 and *S. pseudopneumoniae* type strain ATCC BAA-960 (kindly supplied by Maria de Gloria Carvalho, Division of Bacterial Diseases, Centers for Disease Control and Prevention, Atlanta, GA). In addition, pneumococcal strains D39, G54, 23F, OXC141, INC104B, and INV200 and *S. mitis* NCTC 12261 were used in the comparative genomic hybridization experiments described below.

Antimicrobial susceptibility testing. MICs of penicillin, ceftriaxone, erythromycin, clindamycin, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim were determined with the Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. Results were interpreted following the recommendations and definitions of the Clinical and Laboratory Standards Institute (CLSI) (5).

Detection of antimicrobial resistance genes. Macrolide (*ermB*, *mefA*, or *mefE*) and tetracycline (*tetM*) resistance genes were screened by PCR using primers and conditions previously described (26).

DNA fingerprinting by PFGE. Preparation of chromosomal DNA, digestion with SmaI endonucleases, separation of DNA fragments by pulsed-field gel electrophoresis (PFGE), and interpretation of results were carried out as previously described (40).

Optochin susceptibility. Optochin susceptibility was tested by disk diffusion, using commercially available optochin disks (5 μg; 6 mm; Oxoid, Hampshire, England) applied to blood agar plates (Trypticase soy agar supplemented with 5% sheep blood) inoculated with colonies from overnight cultures. Plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. A similar assay was carried out in ambient atmosphere as described by Arbique et al. (1). Isolates were considered to be resistant to optochin if they displayed inhibition zones smaller than 14 mm.

Capsular typing. Capsular serotyping was performed using the chessboard system (42) with specific antiserum from the Statens Serum Institute (SSI; Copenhagen, Denmark). Omniserum (SSI, Copenhagen, Denmark), a serum that contains antibodies to all known pneumococcal serotypes, was used to confirm nontypeability.

Detection of *cpsA* (a conserved pneumococcal capsular gene present in 89 of the 91 capsular operons described to date) (2) was done by PCR using the primers cpsAF2 (5′-AGCAGTTTGTTGGACTGACC-3′) and cpsAR2 (5′-GTGTGAATGGACGAATCAAC-3′).

lytA **PCR detection, RFLP signatures, and Southern blotting.** PCR was used to screen for the presence of the gene encoding the major autolysin (*lytA*) in *S. pneumoniae*, a virulence factor ubiquitous in pneumococci that is often used as an identification marker of this species, using the primers described by Obregon et al. (32) (LA5_Ext, 5′-AAGCTTTTTAGTCTGGGGTG-3′; LA3_Ext, 5′-AAGCTTTTTCAAGACCTAATAATATG-3′), which yield a PCR product of ca. 1,200 bp encompassing the entire *lytA* gene. Restriction fragment length polymorphism (RFLP) signatures characteristic of typical pneumococcal *lytA* or atypical *lytA* were determined as described before by digesting the PCR product with BsaAI and separating the fragments by agarose gel electrophoresis (24).

For Southern blotting, DNA fragments separated by PFGE were transferred to nylon membranes (41) and hybridized with a *lytA* probe obtained by PCR amplification of TIGR4 DNA with the primers LA5_Ext and LA3_Ext described above.

DOC solubility assays. DOC solubility was initially performed according to standard procedures (38): colonies from an overnight culture were suspended in 1 ml of a 0.85% NaCl (wt/vol) solution to a turbidity equal to 0.5 to 1 McFarland standard. This suspension was distributed into two tubes, and three to four drops of a 10% DOC solution were added to one tube while the other served as a control. Both tubes were incubated at 37°C for up to 2 h. A sample was considered positive when clearing of the turbidity occurred in the tube with DOC but not in the control.

For a more detailed characterization of the isolates, DOC solubility was reassayed in a slightly different way, as described by Llull et al. (24): 1 ml of exponentially growing cultures received 100 μl of 1 M potassium phosphate buffer (pH 8.0) and 100 μl of lysis solution (DOC at a final concentration of 1% or 0.1%). The mixtures were incubated for 15 min at 37°C. Turbidities of the solutions were read at 620 nm using an Ultrospec III instrument (Pharmacia LKB, Cambridge, United Kingdom). When the turbidity of the cell suspension decreased more than 50% from the initial value, the assay was considered positive. The experiments were repeated three times on different days.

ply **and** ***mly*** **PCR detection and BsaI-RFLP signatures.** The presence of *ply* (encoding pneumolysin, a cholesterol-dependent citolysin) or *mly* (a *ply* homologue recently identified in some *S. mitis* isolates that has been named mitilysin) (16) was detected by PCR. The primers used for detection of both genes were plyF (5′-TTCTGTAACAGCTACCAACG-3′) or plyF2 (5′-CGATGAGTTTGTTGTTATCG-3′) with plyR (5′-ACCTTATCYTCTACCTGAGG-3′), yielding an internal fragment of 1,223 bp with primer plyF and 1,283 bp with primer

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plyF2. Alignment of the sequences of *ply* and *mly* alleles available at NCBI (accession numbers EF413923 to EF413926, EF413929 to EF413931, EF413933, EF413934, EF413936, EF413937, EF413939, and EF413943 for *ply* alleles and EF066514 to EF066520 for *mly* alleles) showed that the PCR products obtained for these genes could be distinguished by differential restriction sites for BsaAI; BsaAI cuts the *ply* PCR product once, resulting in two fragments of 830 bp and 393 bp (or 890 bp and 393 bp), while the *mly* PCR product does not contain a restriction site for BsaAI. Thus, after purification of the PCR products, the fragments were digested with BsaAI and separated by agarose gel electrophoresis, and the presence of *ply* or *mly* was inferred from the pattern obtained.

Screening for pneumococcus-specific lipoprotein *piaA*. Detection of *piaA*, a gene encoding the lipoprotein component of the pneumococcal iron ABC transporter Pia and described as 100% conserved and uniquely found in pneumococci (47), was done by PCR. The primers used were those previously described: piaAFor (5′-AGAGCATGCGCCTGATAAAAT-3′) and piaARev (5′-CATGAGGCTGCTAACGGTGTAT-3′) (47).

Multilocus sequence typing. Amplification of internal fragments of seven housekeeping genes—*aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*—was done according to the MLST scheme developed by Enright and Spratt for *S. pneumoniae* (6). Sequencing reactions were conducted at Macrogen, Inc. (Seoul, South Korea). Sequencing analysis was done with DNAStar (Lasergene). Tentative allele number assignment was done at the international MLST database for *S. pneumoniae* (www.mlst.net).

Multilocus sequence analysis. Phylogenetic analysis of MLST data was done by concatenating the sequences of all MLST loci except *ddl* to obtain one single sequence of 2,758 bp (9). One isolate failed to yield an amplification product for a single locus (*gdh* for PT5645a), and, in this case, a shorter contig was constructed. MLST sequences of *Streptococcus* spp. including *S. mitis*, *S. pseudopneumoniae*, and *S. oralis*, previously described by Hanage et al. and Chi et al. (4, 9) were also used. Sequence alignment was performed using the ClustalW algorithm included in MEGA, version 4, build 4025 (43). A bootstrap consensus tree inferred from 1,050 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method.

Comparative genomic hybridization. Microarrays used in this study have been described in detail before and contain PCR amplicons representing 2,087 open reading frames (ORFs) of *S. pneumoniae* TIGR4, all spotted in duplicate (10). Chromosomal DNA for CGH was isolated from bacterial cultures by cetyltrimethylammonium bromide extraction as described previously (44). In all cases, TIGR4 genomic DNA was used as the reference sample. For CGH, 400 ng of DNA from each strain was fluorescently labeled using the BioPrime Array CGH Genomic Labeling System (Invitrogen). The concentrations of nonfluorescent nucleotides in the 50-μl reaction mixtures were 0.2 mM each of dATP, dCTP, and dGTP and 0.1 mM dTTP, and fluorescent nucleotide analogs (Cy5-dUTP or Cy3-dUTP; Amersham Biosciences) were added to a final concentration of 0.1 mM. The reaction mixture was incubated overnight at 37°C, and the reaction was stopped by addition of 5 μl of 0.5 M EDTA (pH 8.0). Reactions were cleaned up using the purification module of the BioPrime kit, after which yields and incorporation of dye were verified using a Nanodrop ND-1000 (Nanodrop Technologies). Labeled sample and reference DNA were combined and precipitated with ethanol in the presence of 0.3 M sodium acetate (NaAc; pH 5.2). Dried samples were resuspended in 65 μl of Slidehyb buffer 1 (Ambion) and applied to the microarrays underneath a lifter slip (Erie Scientific Co.). Following overnight hybridization at 45°C, arrays were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; Invitrogen) containing 0.25% SDS for 5 min, followed by two washes in 1× SSC and 0.5× SSC for 5 min each. Finally, slides were dipped into H_2O and dried by centrifugation. Two replicate hybridizations (dye swap) were performed for all strains.

CGH data acquisition and analysis. Dual-channel array images were acquired on a GenePix 4000AL microarray scanner and analyzed with GenePix Pro software (Axon Instruments, Union City, CA). Automatically flagged spots, spots with low-background-subtracted signal intensities (sum of median Cy3 and Cy5 net signals of <1,000) and spots with >40% saturated pixels were filtered out of all data sets prior to analysis. Slides used for CGH of pneumococcal strains were normalized by an array-based Lowess transformation. Due to the highly skewed distribution of log ratios with the atypical strains, array-based Lowess transformation could not be performed with these arrays. Instead, we selected a subset of genes (list available on request) that gave consistent hybridization signals for all strains and calculated a normalization factor so that the mean ratio across this gene set was 1. Next, average normalized log ratios were calculated for genes with at least three measurements per strain in the dye swap pair. As a final selection criterion, only genes with valid data for 15 out of the 25 atypical strains (total, 1,838) were used. Designation of genes in each strain as present, divergent, or absent was performed using the graded assignment categorization option of the GACK software (22). GACK uses the ratio distribution per strain to calculate an estimated probability of presence (EPP) value for each gene without the need for arbitrarily defined cutoffs. A calculated EPP of 100% indicates that a gene is present (assigned 0.5), an EPP of 0% indicates a gene is absent (assigned −0.5), and intermediate EPP values indicate that a gene is divergent (assigned a value between −0.5 and 0.5 on a linear scale, where a value of 0 indicates that a gene has a 50% chance of being divergent or present). Clustering was based on the GACK scores and was done with the Euclidian distance metric and average linkage, using TIGR Multiexperiment Viewer (MeV; http://www.tm4.org/mev.html).

Analysis of *comC* allelic variation. Chromosomal DNA from each strain was obtained using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions. *comC* PCR products of 337 bp were obtained as previously described by Whatmore and coworkers (48) using the primers comC_f (5′-TGACAGTTGAGAGAATCTT-3′) and comC_r (5′-CTTTTCTATTTATTTGACCT-3′). Sequencing was conducted at Macrogen, Inc. (Seoul, South Korea), and subsequent analysis of obtained sequences was done with DNAstar (Lasergene).

RESULTS

Presumptive identification of atypical streptococci. The 25 strains described in this study were all isolated from selective agar medium (containing gentamicin) routinely used to recover pneumococci while inhibiting the growth of other bacterial species that colonize the nasopharynx. All strains displayed a pneumococcus-like colony morphology, and although they were resistant to optochin, they were bile soluble. Therefore, they were presumptively identified as optochin-resistant pneumococci.

Abnormally high levels and prevalence of penicillin and multidrug resistance. Antimicrobial susceptibility testing showed that all but one of the 25 strains were nonsusceptible to penicillin (MIC higher than 0.06 mg/liter), and 64% were multidrug resistant (defined as nonsusceptibility to three or more classes of antimicrobial agents) (Table 1). Among the 19 isolates resistant to macrolides, 3 contained *ermB*, 14 contained either *mefA* or *mefE*, and two contained both *ermB* and either *mefA* or *mefE*; the 7 isolates resistant to tetracycline contained *tetM* (Table 1). Furthermore, penicillin MICs of 15 strains were high, ranging from 2 to 6 mg/liter, values that are extremely rare in Portuguese colonization samples and could have important implications. These results prompted a detailed characterization to clarify whether the 25 strains were true pneumococci.

Phenotypic characterization. No pneumococcal capsule could be detected in any of the strains by the Quellung reaction: eight isolates showed no positive reaction, and the remaining ones autoagglutinated. In addition, *cpsA* could not be detected by PCR in any of the strains, suggesting its absence.

To determine if the isolates showed the CO_2-dependent optochin phenotype described for *S. pseudopneumoniae* (1), we examined optochin susceptibility in 5% CO_2 and ambient atmosphere in parallel. Twenty isolates were fully resistant to optochin in both environments (no halos were obtained). Four isolates (PT5295b, PT5557b, PT5645, and PT5729) had increased susceptibility in ambient atmosphere but were still considered resistant according to the proposed interpretation criteria. One isolate (PT5479) had results identical to those described for *S. pseudopneumoniae*; i.e., it was resistant to

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TABLE 1. Properties of strains characterized in this study

Isolate	DCC code	Child's age	Bile solubility[a]	MIC (mg/liter)[b]							Presence of resistance gene(s)	PFGE pattern	*lytA*[c]		Presence of *ply* or *mly*	MLST allele assignment[d]						
				PenG	Tx	Ery	Cc	Tet	Chl	SXT			RFLP	Southern blotting[a]		*aroE*	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
PT5274	16	5	Pos	**0.75**	0.25	**>256**	0.125	**8**	3	**3**	*ermB*, *tetM*	1	Atypical	Pos	*mly*	P	O	A	Q	S	G	G
PT5283	16	4	Pos	**6**	**0.75**	**8**	0.094	0.25	2	**1**	*mefA* or *mefE*	2	NA	Pos	*mly*	N	T	181	N	T	T	A
PT5295b	32	4	Pos	**2**	0.38	**24**	0.094	0.38	2	**2**	*mefA* or *mefE*	3	NA	Pos	*mly*	R	I	G	O	U	U	K
PT5346b	32	5	Pos	**0.5**	0.19	**24**	0.094	**32**	2	**0.75**	*mefA* or *mefE*, *tetM*	4	Atypical	Pos	*mly*	U	A	F	E	K	V	I
PT5479	20	5	Neg	**0.38**	0.25	0.094	0.094	0.19	2	0.064		5	Atypical	Neg	*mly*	X	J	O	B	D	C	6
PT5525b	35	3	Pos	**4**	0.5	**16**	0.064	0.38	2	**1.5**	*mefA* or *mefE*	6	NA	Pos	*mly*	V	T	P	F	V	N	A
PT5525c	35	3	Pos	**0.094**	0.094	**16**	0.064	0.38	3	**3**	*mefA* or *mefE*	7	Atypical	Pos	*mly*	A	B	D	C	L	I	S
PT5532	35	4	Pos	**0.25**	0.25	**>256**	**>256**	**32**	3	**6**	*ermB*, *tetM*	8	Atypical	Pos	*mly*	L	R	D	J	A	D	S
PT5534b	35	5	Pos	**4**	**2**	**12**	0.094	0.25	2	0.5	*mefA* or *mefE*	9	—	Pos	*ply*	Q	K	L	K	E	E	H
PT5557b	35	5	Pos	**3**	0.38	**24**	0.064	0.38	2	**1**	*mefA* or *mefE*	6	NA	Pos	*mly*	F	T	181	F	I	N	A
PT5590	19	4	Pos	**3**	**0.75**	**16**	0.094	0.38	2	0.19	*mefA* or *mefE*	10	Atypical	Pos	*ply*	J	E	A	S	W	W	G
PT5590b	19	4	Pos	**6**	0.5	**48**	**>256**	1	3	**3**	*ermB*, *tetM*	11	NA	Pos	*mly*	T	P	S	H	G	R	L
PT5645	21	4	Pos	**6**	0.38	**16**	0.094	0.38	2	**1.5**	*mefA* or *mefE*	12	NA	Pos	*mly*	M	na	M	I	P	L	D
PT5645b	21	4	Pos	0.064	0.064	0.064	0.094	0.25	3	0.094		13	Atypical	Pos	*ply*	K	S	J	G	Q	S	102
PT5714	21	3	Pos	**0.25**	0.064	**16**	0.094	0.38	2	0.125	*mefA* or *mefE*	14	Atypical	Pos	*mly*	D	Q	H	G	F	A	J
PT5729	21	3	Pos	**0.5**	0.125	**12**	0.094	**24**	2	**1.5**	*mefA* or *mefE*, *tetM*	15	NA	Pos	*mly*	E	C	R	F	N	F	R
PT5736b	21	3	Pos	**0.38**	0.04	**32**	0.094	**32**	2	0.125	*mefA* or *mefE*, *tetM*	16	Atypical	Pos	*mly*	C	94	K	R	O	B	B
PT5779	22	3	Pos	**0.25**	0.094	**16**	0.094	0.38	3	**6**	*mefA* or *mefE*	17	Atypical	Pos	*ply*	I	D	E	H	C	H	F
PT5787b	22	2	Pos	**3**	**0.75**	0.064	0.064	0.25	2	**6**		18	Atypical	Pos	*mly*	W	H	A	A	M	Q	M
PT5790b	22	0	Pos	**3**	**0.75**	0.094	0.094	0.38	2	**6**		18	Atypical	Pos	*mly*	S	H	A	A	M	P	M
PT5793b	22	0	Pos	**3**	0.5	**>256**	**>256**	**32**	4	**3**		19	Atypical	Pos	*mly*	B	F	I	P	J	O	O
PT5794b	22	0	Pos	**2**	**0.75**	0.064	0.064	0.25	3	**3**	*ermB*, *mefA* or *mefE*, *tetM*	20	Atypical	Pos	*mly*	W	H	A	A	M	22	P
PT5796b	22	2	Pos	**6**	**0.75**	0.094	0.094	0.25	3	**3**		21	Atypical	Pos	*mly*	1	L	B	D	B	K	C
PT5798b	22	2	Pos	**3**	**1**	**16**	0.125	0.25	2	**2**	*mefA* or *mefE*	22	Atypical	Pos	*mly*	O	N	N	M	161	J	Q
PT5804	22	0	Pos	**2**	0.38	**>256**	**>256**	**24**	3	**1**	*ermB*, *mefA* or *mefE*, *tetM*	23	Atypical	Pos	*ply*	G	G	Q	L	R	P	C

[a] Neg, negative; Pos, positive.
[b] MICs in boldface indicate nonsusceptibility (intermediate and resistant). PenG, penicillin; Tx, ceftriaxone; Ery, erythromycin; Cc, clindamycin; Tet, tetracycline; SXT, sulfamethoxazole-trimethoprim; Chl, chloramphenicol.
[c] —, no RFLP could be obtained although a PCR product with expected size was amplified; NA, PCR product could not be obtained.
[d] MLST alleles were assigned capital letters when they were not described in the MLST pneumococcal database.

optochin when incubated in 5% CO_2 but susceptible when incubated in ambient atmosphere.

Deoxycholate solubility assays at 1% and 0.1% final concentrations following the protocol proposed by Obregon et al. (32) were carried out. These authors reported that streptococci of the mitis group (SMG) harboring an atypical *lytA* did not lyse with 1% DOC solution but lysed at 0.1%; i.e., in the latter case the turbidity of the cell suspension decreased at least 50% after 15 min at 37°C. In our study, all but one strain lysed in the presence of both concentrations of DOC, a result reminiscent of typical pneumococci and similar to control strains R6 and TIGR4. The only exception found (PT5479) was the strain that, based on optochin susceptibility, was tentatively classified as *S. pseudopneumoniae.* The same behavior was observed for *S. pseudopneumoniae* control strain ATCC BAA960.

Screening for pneumococcus-specific genes. Screening of *lytA* by PCR yielded a product with the expected size in 18 isolates (Table 1). By BsaI-RFLPs, a signature identical to the atypical *lytA* found in some streptococci of the mitis group was obtained for 17 isolates (data not shown) (24). For one strain (PT5534b) a restriction pattern could not be obtained (Table 1). In the remaining seven isolates no PCR product could be obtained despite several repeated attempts under different experimental conditions. However, by Southern blotting of SmaI-PFGE patterns, in all but one strain (PT5479), at least one fragment hybridized with a *lytA*-specific probe, suggesting that some form of this gene was present (Table 1).

PCR screening for *ply* or *mly* indicated that all 25 strains harbored one of these allelic variants. Subsequent BsaAI-RFLP analysis indicated that five isolates harbored pneumolysin and 20 harbored mitilysin (Table 1). PCR screening for the *piaA* gene suggested that it was absent in all 25 strains characterized in this study.

Genotyping by PFGE and MLST/MLSA. PFGE analysis showed a high level of diversity between the 25 atypical strains as 23 distinct PFGE patterns were identified, and only two pairs of strains displayed the same profile (Table 1).

Sequence analysis of the seven housekeeping genes included in the *S. pneumoniae* MLST scheme showed that among the 174 alleles obtained, only 7 had been previously deposited in the pneumococcal database (i.e., the alleles that are assigned numbers in Table 1). All other alleles diverged up to 9% from the closest available match. The simultaneous occurrence of six to seven novel alleles in each strain suggested that they were not authentic pneumococci (9).

The phylogenetic tree resulting from the MLSA clearly placed the strains described in this study apart from *S. pneumoniae* and *S. oralis* (Fig. 1). In particular, all strains but one clustered together with the isolates of the streptococci of the mitis group described by Chi et al. (4). However, among the SMG, the strains described here formed a separate group that diversified close to the root of the tree. One strain, PT5479, clustered together with several *S. pseudopneumoniae* isolates described by Hanage et al. (9).

Genomic characterization by CGH. To obtain insights into the variation of gene content between the isolates described in this study and *S. pneumoniae*, we performed CGH using *S. pneumoniae* TIGR4 as a reference strain. In addition to the 25 atypical isolates, we also used seven true pneumococcal strains (six clinical isolates and strain R6), *S. mitis* NCTC 12261, and *S. pseudopneumoniae* ATCC BAA960. Clustering of strains based on the GACK absent/present/divergent gene scores for the 1,838 TIGR4 genes that passed our selection criteria showed that all 25 atypical strains clustered together and that they appeared to be more similar to streptococcus of the mitis group than to the cluster of true pneumococcal strains, consistent with the MLSA findings. CGH also confirmed the close relationship of the two strain pairs with identical PFGE pattern, PT5525b/PT5557b, and PT5787b/PT5790b. A few gene clusters appeared to be absent or divergent in all strains examined, including the pneumococcal strains such as the TIGR4-specific capsule locus, the *rlrA* pathogenicity islet, a cluster of hypothetical ORFs, and a fructose phosphotransferase system (PTS).

Of the 1,838 genes used for the phylogenetic analysis, 1,365 belonged to the pneumococcal core genome proposed by Obert et al. (31). CGH analysis indicated that 394 pneumococcal core genes were divergent or absent in at least one of the 25 atypical isolates, with an average of 157 per strain. In total, 53 core genes were divergent or absent in all 25 isolates. In *S. pseudopneumoniae* and *S. mitis*, respectively, 135 and 318 core genes were absent or divergent. Interestingly, most of the absent or divergent core genes were grouped into 12 TIGR4 chromosomal regions of diversity (see Table S1 in the supplemental material). These genes are predicted to encode proteins with a variety of functions, but most prominent classes are involved in transport (e.g., ABC transporters and PTS systems) and energy metabolism.

To assess whether the atypical strains contained factors known to be important for the infection potential of *S. pneumoniae*, we focused on the CGH results for some of the major pneumococcal virulence genes (Fig. 2). As already demonstrated by the PCR-RFLP analysis described above, *ply* or *mly* (*ply*-like) sequences could be detected in all of the atypical strains. The *lytA* gene was found to be present in 11 of the 25 strains while it was classified as absent or divergent in the remainder, including five of the seven strains that failed to yield a *lytA* PCR product. Major differences between *S. pneumoniae* and the atypical strains appeared to reside in the PiaA iron ABC transporter system (as was observed by PCR) and in genes encoding the choline-binding protein surface proteins CbpG and PcpA and the neuraminidases NanA and NanB (Fig. 2). By contrast, other genes involved in virulence in *S. pneumoniae*, such as *pavA*, *spxB*, *cbpE*, and *cbpD*, were found to be present in the atypical isolates as well.

Allelic variation of competence stimulating peptide (*comC*). Most members of the mitis group are naturally competent for genetic transformation, and CGH indicated that the known competence genes were indeed present in our isolates. To examine potential sequence diversity of *comC* in more detail, we sequenced a *comC* PCR product from 23 strains. A total of 14 different alleles based on the amino acid sequence of ComC were identified. Of these, only four have been previously described (Table 2) (21, 35).

DISCUSSION

This study describes several phenotypic and genotypic properties of a collection of streptococcal isolates obtained in a single cross-sectional study aimed to describe the impact of

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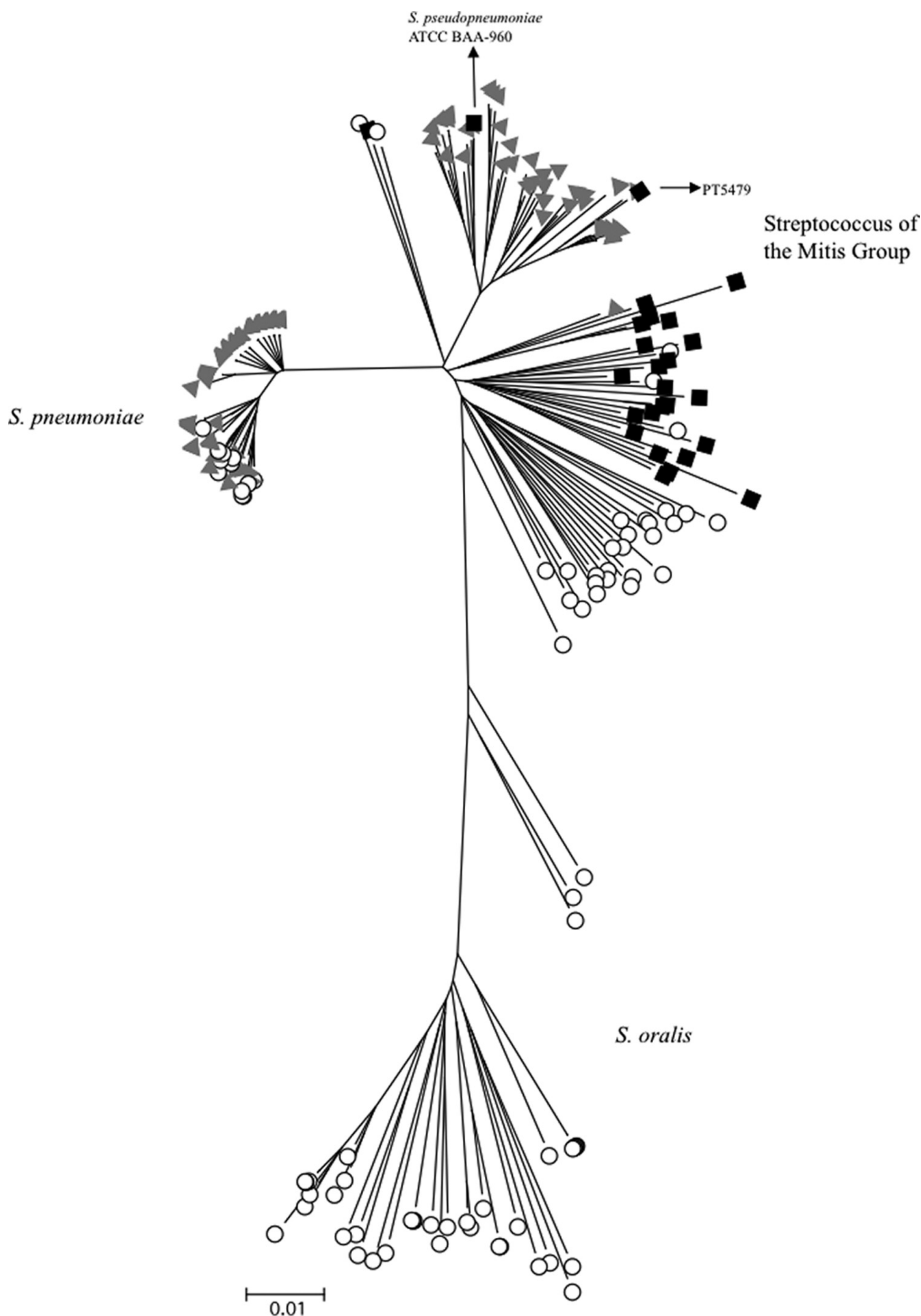

FIG. 1. Genetic relationships of the strains determined by MLSA. Black squares, strains described in this study; gray triangles, strains described by W. Hanage (9); white circles, strains described by F. Chi (4) (details in text).

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PCV7 in colonization. Based on routinely used assays such as colony morphology, bile solubility, and optochin susceptibility, these isolates were initially classified as optochin-resistant, bile-soluble pneumococci. However, they did not agglutinate with anti-pneumococcal capsular antibodies, and several isolates displayed unusually high MICs to penicillin (2 to 6 mg/liter), indicating a sudden increase in the prevalence of penicillin resistance from 1.7% to 5% over a period of 3 years.

Further attempts to characterize these isolates by PCR screening for *lytA* and *plyA* were not conclusive as most strains appeared to have some form of *lytA* and *plyA*. Additional analyses showed that the isolates contained atypical *lytA* genes, as defined by Llull et al. (24); five had pneumolysin, the others had the recently described mitilysin (16), and all lacked *cpsA* and *piaA*. However, the latter two genes are known to be absent in some noncapsulated true pneumococci (47), and,

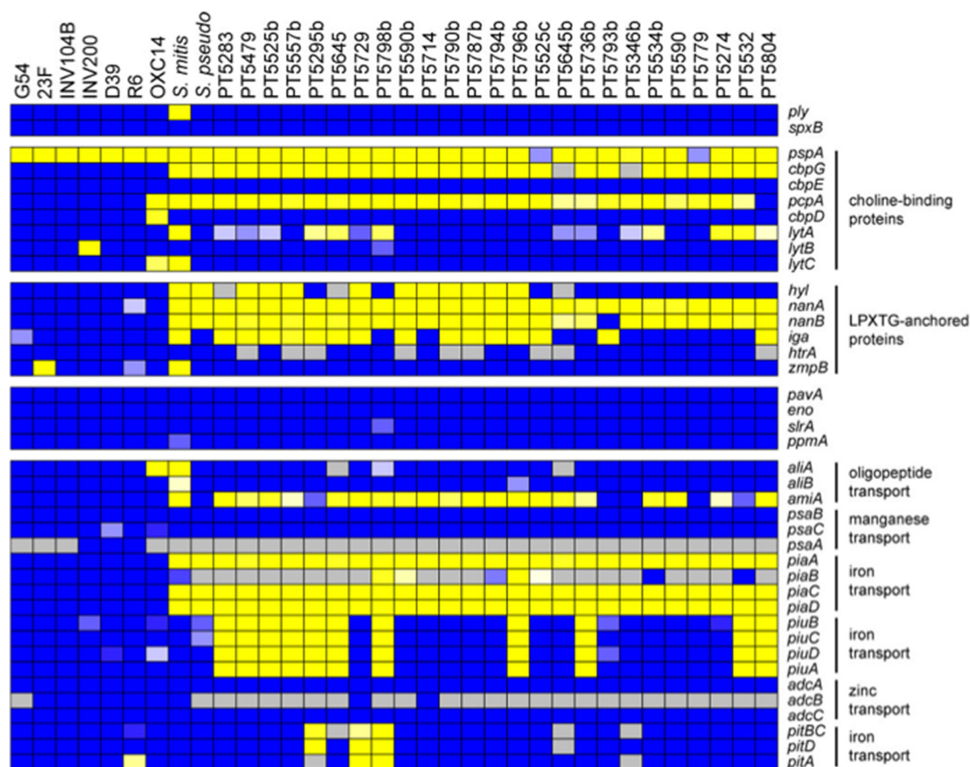

FIG. 2. Gene content variation in pneumococcal virulence factors. CGH data for selected genes encoding major pneumococcal virulence factors are shown. The colors indicate the status of the ORFs, as follows: blue, present; yellow, absent; gray, no data.

thus, their usefulness in defining the species is limited when they are absent. DNA fingerprinting by PFGE indicated that the strains were genetically diverse. Epidemiological data also suggested that they were mostly unrelated as they came from seven different day care centers.

Isolates distinct from pneumococci but that cannot be resolved from them by optochin susceptibility, bile solubility, or the presence of genes such as *lytA* and *plyA*, have been described in the literature (9, 14, 23, 32, 45, 49).

To establish the relationship between our strains and true pneumococci, we characterized them by MLSA and CGH and compared results with those obtained for control strains of *S. pneumoniae*, *S. pseudopneumoniae*, *S. mitis*, and *S. oralis*. On the basis of MLSA, the isolates were identified as streptococci of the mitis group, a classification that has been proposed for a group of evolutionarily related streptococcal isolates for which species classification challenges currently accepted criteria (32). In the SMG group, one cluster included several *S. pseudopneumoniae* isolates described by Hanage et al. and strain PT5479 isolated in this study (9). The latter was the single strain that had been identified as *S. pseudopneumoniae* based on optochin susceptibility assays. CGH also indicated

TABLE 2. *comC* alleles of strains characterized in this study

Isolate	Amino acid sequence of *comC*[a]	Reference	Allele/strain
PT5714	MKNTVKLEQFVALKEKDLQNIKGG**ESRISDILLDFLFQRKK**	35	CSP8
PT5590	MKNTVKLEQFVALKEKDLQNIKGG**ESRISDILLGFLFQRKK**		
PT5479, PT5779	MKNTVKLEQFVALKEKDLQKIKGG**EMRLPKILRDFIFPRKK**	35	CSP6.1
PT5274, PT5525c, PT5736b, PT5787b, PT5790b, PT5794b	MKNTVKLEQFVALKEKDLQKIKGG**ESRLSRLLRDFIFQIKQ**	21	SK612
PT5645	MKNTVKLEQFVTLKEKDLQKIQGG**ESRLSRLLRDFIFQIKQ**		
PT5798b	MKNTVKLEQFVALKEKDLQKIQGG**ESRLSRLLRDFIFQIKQ**		
PT5283	MKNTVKLEQFVALKEKDLQKIKGG**ESRMPKILRDFIFPRKK**		
PT5295b, PT5525b, PT5557b	MKNTVKLEQFVTLKEKDLQKIKGG**ESRMPKILRDFIFPRKK**		
PT5804	MKNTVKLEQFVTLKEKDLQEIRGG**ESRMSKFLLDFLFQRKK**		
PT5346b	MKNTVKLEQFVALKEKDLQEIRGG**ESRVSRIILDFLFLRKK**	21	SK675
PT5729	MKNTVKLEQFVALKEKDLQEIQGG**ESRLSKLLRDFILQRKK**		
PT5590b	MKNTVKLEQFVTLKEKDLQEIQGG**EMRKKIESFPGIFNFFRRR**		
PT5532, PT5796b	MKNTVKLEQFVALKEKDLQNIQGG**EMRKMNEKSFNIFNFFRRR**		
PT5645b	MKNAVKLEQFVSLKEKDLQKIKGG**DMRKKIESFPGLFNFFRRR**		

[a] Sequence of the mature CSP is given in boldface.

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that the atypical strains are indeed distinct from true pneumococci. However, it showed that a considerable part of the proposed pneumococcal core genome (88% on average) is conserved in these isolates. Absent regions corresponded mainly to loci encoding proteins involved in nutrient uptake and metabolism. Interestingly, many of the known pneumococcal virulence factors were detected by CGH, with a few notable exceptions: CbpG, a surface protein that functions as an adhesin to eukaryotic cells, the neuraminidases NanA and NanB, and the Pia iron-uptake system.

Why species definition is sometimes difficult (if not impossible) for some nonhemolytic streptococcal isolates is a matter of debate. Killian et al. (21) have recently proposed that the ancestor of the pneumoniae-mitis-pseudopneumoniae group was a pneumococcus-like bacterium with all the properties associated with virulence. The commensal streptococci subsequently evolved from this pathogen by genome reduction, thus explaining the random (but syntenic) presence of pneumococcal virulence genes in representatives of this group. Others have suggested that horizontal gene transfer between naturally competent streptococci that share the same ecological niche significantly contributes to attenuation of putative barriers between these species, resulting in not only mosaic genes but also mosaic genomes (1, 4, 7, 8). While our results based on CGH do not enable us to favor any of these theories, they do enhance our perception of how closely related these species are.

Indeed, known competence genes required for natural transformation were detected in the atypical isolates by CGH. Furthermore, several different *comC* alleles, coding for the competence stimulating peptide (CSP), were found by sequence analysis, most of which appeared to be novel. Cross-communication between isolates requires recognition of the CSP by a ComD receptor. Cross talk between different alleles due to promiscuous ComD has been described (17). Whether that is the case for the isolates described in this study has not been investigated but could potentially contribute to the ambiguous nature of these strains.

High rates of resistance to penicillin and high MICs are frequent among *S. mitis* isolates (13, 32, 33). Therefore, misidentification of SMG isolates such as those described here can result in overreported levels of penicillin resistance, a problem previously identified (36, 46). In our study, after ruling out the 15 SMG isolates, penicillin resistance levels among pneumococci were found to have remained stable over time.

It is unclear why we have identified these SMG isolates only now, as we have been conducting surveillance studies since 1996. Most (15 of 25) were picked from the primary selective blood agar plate as secondary isolates that displayed colony morphologies distinct from the majority of the sample but still resembled pneumococci. Our recent focus on noncapsulated pneumococci and optochin resistance, as well as an interest in multiple colonization, most likely contributed to an increased propensity to pick such colonies. Alternatively (and not mutually exclusively), it could be that changes in the nasopharynx ecosystem due to introduction of the seven-valent pneumococcal conjugate vaccine facilitated colonization by SMG, thereby increasing its abundance in the population. The prevalence of these “confounding” organisms remains unknown, and their contribution to pneumococcal evolution remains to be ascertained.

In conclusion, in the era of multivalent pneumococcal conjugate vaccines where surveillance is very important, researchers need to be aware that routinely used tests to identify pneumococci may not be sufficient when atypical isolates are found. This is critical in colonization studies since closely related streptococci inhabit the same niche and can be highly resistant to antibiotics. On the other hand, although *S. pseudopneumoniae* has been implicated in chronic obstructive pulmonary disease (COPD) (19), it is not clear whether the atypical confounding organisms described in this study may cause disease. This will be the subject of future research, aiming at increasing the insight into the ecological and clinical roles of these strains.

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We report that we have no conflicts of interest.

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