Identification of the *cpsA* gene as a specific marker for the discrimination of *Streptococcus pneumoniae* from viridans group streptococci

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Streptococcus pneumoniae, the aetiological agent of pneumonia and non-gonococcal urethritis, shares a high degree of DNA sequence identity with the viridans group of streptococci, particularly *Streptococcus mitis* and *Streptococcus oralis*. Although their clinical and pathological manifestations are different, discrimination between *S. pneumoniae* and its close viridans cocci relatives is still quite difficult. Suppression subtractive hybridization was performed to identify the genomic differences between *S. pneumoniae* and *S. mitis*. Thirty-four resulting *S. pneumoniae*-specific clones were examined by sequence determination and comparative DNA sequence analysis using BLAST. *S. pneumoniae*-specific primers were subsequently designed from one of the clonal DNA sequences containing the *cps* gene (coding for capsular polysaccharide biosynthesis). The primer specificities were evaluated using 49 viridans streptococci including 26 *S. pneumoniae*, 54 other streptococci, 14 *Lactococcus* species, 14 *Enterococcus* species and three *Vagococcus* species, and compared with the specificities of previously described autolysin (*lytA*), pneumolysin (*ply*), Spn9802 and Spn9828 primers. The newly designed *cpsA*-specific primer set was highly specific to *S. pneumoniae* and was even better than the existing primers. These findings may help improve the rapid identification and differentiation of *S. pneumoniae* from closely related members of the viridans group streptococci.

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INTRODUCTION

Streptococcus pneumoniae is an important pathogen associated with various human diseases such as pneumonia, non-gonococcal urethritis, otitis media, septicemia and meningitis (Whatmore *et al.*, 2000). In contrast, two viridans group streptococci, *Streptococcus oralis* and *Streptococcus mitis*, cause dental caries and

endocarditis and are usually considered commensal micro-organisms of the oral cavity (Dyson *et al.*, 1999; Willcox *et al.*, 1988). Because *S. pneumoniae* is often isolated with *S. mitis* and *S. oralis*, accurate discrimination between these strains is important for proper diagnosis and treatment of *S. pneumoniae*.

In recent years, molecular genetic analyses based on the 16S rRNA gene have provided a potent means of characterization at the species level (Fox *et al.*, 1992; Stackebrandt *et al.*, 1991; Stackebrandt & Goebel, 1994). Unfortunately, *S. pneumoniae* shares over 99 % 16S rRNA gene identity with

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Abbreviation: SSH, suppression subtractive hybridization.

S. mitis and *S. oralis* (Suzuki *et al.*, 2005), complicating molecular identification among them. Several molecular assays have been developed to identify *S. pneumoniae* from the other viridans group streptococci using primers specific to pneumococcal virulence factors such as genes encoding pneumolysin (*ply*) (Corless *et al.*, 2001), autolysin (*lytA*) (McAvin *et al.*, 2001), pneumococcal surface antigen A (Morrison *et al.*, 2000), manganese-dependent superoxide dismutase (Kawamura *et al.*, 1999) and penicillin-binding protein (O'Neill *et al.*, 1999).

Recently, real-time PCR methods have improved the detection of these organisms (Abdeldaim *et al.*, 2008; Carvalho *et al.*, 2007; Corless *et al.*, 2001; Suzuki *et al.*, 2006). At the same time, increasing evidence suggests the possible occurrence of lateral gene transfer among the naturally transformable viridans group streptococci. Indeed, some pneumococcal virulence genes of *S. pneumoniae* have occasionally been detected in *S. mitis* or *S. oralis* isolates (Seki *et al.*, 2005; Verhelst *et al.*, 2003; Whatmore *et al.*, 2000).

Previously, two genomic clones specific to *S. pneumoniae*, referred to as Spn9802 and Spn9828, were identified by genomic subtractive hybridization (Suzuki *et al.*, 2005). A few false-positive results were reported, however, from samples where *S. pneumoniae* was not isolated (Abdeldaim *et al.*, 2008; Suzuki *et al.*, 2006).

Suppression subtractive hybridization (SSH) is a powerful tool for searching for genomic differences among closely related strains (Kim *et al.*, 2008) or for identifying novel virulence factors by genomic comparison of pathogenic and non-pathogenic strains (Agron *et al.*, 2002). In this study, SSH was used to identify novel genomic markers specific to *S. pneumoniae*, allowing accurate discrimination of *S. pneumoniae* from members of the closely related viridans group streptococci.

METHODS

Bacterial strains. A total of 134 bacterial strains were used in this study (Table 1). The 26 *S. pneumoniae* strains were obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea), the Culture Collection of Antibiotic-resistant Microbes (CCARM; Seoul, Korea) and the Department of Oral Biochemistry, College of Dentistry, Chosun University (ChDC; Gwangju, Korea). The remaining 108 reference *Streptococcus* strains were obtained from the Culture Collection of the University of Gothenburg (CCUG; Gothenburg, Sweden), the American Type Culture Collection (ATCC; Manassas, VA, USA), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany), the Belgian Co-ordinated Collections of Micro-organisms (BCCM/LMG; Gent, Belgium), the ChDC or the KCTC.

Genomic DNA preparation. Each bacterial strain was grown on either 5% sheep blood agar (Difco) for oral streptococci strains or brain–heart infusion agar (Difco) for the other strains at 37 °C for 20 h. Genomic DNA was extracted from 1.4 ml bacterial culture using a cetyltrimethylammonium bromide method (Ausubel *et al.*, 1993). Purified DNA was dissolved in sterile water with 40 µg RNase

A ml⁻¹ and quantified using an Infinite 200 NanoQuant (Tecan) at a wavelength of 260 nm.

Construction of the SSH library. SSH was performed with *S. pneumoniae* KCTC 5080^T as the tester genomic DNA and the most closely related *Streptococcus* species, *S. mitis* KCTC 3556^T, as the driver genomic DNA. The SSH library was generated using a PCR-Select Bacterial Genome Subtraction kit (Clontech) as described previously (Akopyants *et al.*, 1998). Briefly, both tester and driver genomic DNAs were digested with the restriction enzyme *RsaI* (New England Biolabs) at 37 °C for 2 h to obtain DNA fragments smaller than 3.0 kb. Two different adaptors were separately ligated to the 5' end of the restricted tester DNA fragments: one with adaptor 1 (5'-CTAATACGACTC-CTATAGGGCTCGAGCGGCCGCCCGGCAGGT-3' and 3'-GGC-CCGTCCA-5') and the other with adaptor 2R (5'-CTAATACGACTC-CACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3' and 3'-GCCG-GCTCCA-5'). Two hybridizations were performed. In the first hybridization, the restricted driver DNA fragments were added to each adaptor-linked tester DNA fragment (ratio 4:1). The resultant two driver/adaptor DNA mixtures were denatured at 98 °C for 1.5 min, annealed at 42 °C for 1.5 h and then mixed together before the second hybridization. Five hundred nanograms of the driver genomic DNA was added to the combined DNA mixture before the second hybridization, which was carried out at 42 °C for 16 h. Using the adaptor-specific primer (5'-CTAATACGACTCACTATAGGGC-3'), the tester-specific DNA fragments were amplified by two sequential nested PCRs, as only the unhybridized tester DNA sequences should reassociate to form double-stranded tester DNA fragments ligated to two different adaptors. The resultant PCR products were ligated into the pCR2.1-TOPO vector system (Invitrogen) and transformed into One Shot TOP10 *E. coli* strains (Invitrogen). The transformants were plated onto Luria–Bertani medium containing ampicillin/IPTG/X-Gal and white colonies were screened for the insert fragment. Colony PCR with the nested primers 1 (5'-TCGAGCGGCCGCCCGGCAGGT-3') and 2R (5'-AGCGTGGTCGCGGCCGAGGT-3') was used to confirm the presence of the insert DNA fragments by agarose gel electrophoresis.

Reverse Southern dot-blot analysis. All putative clones were subjected to differential dot-blot analysis to verify whether they were specific for the tester genomic DNA. Briefly, 25 µl of equal amounts of each DNA sample were dotted in duplicate onto positively charged Hybond-N⁺ nylon membranes (Amersham Biosciences). DNA samples were exposed to 1000 mJ UV for 3.5 min in a Stratalinker (Stratagene). DNA probes were prepared with the *RsaI*-digested genomic DNA of each strain (*S. pneumoniae* KCTC 5080^T and *S. mitis* KCTC 3556^T) using an ECL Direct Nucleic Acid Labelling and Detection System (Amersham Biosciences). The probe was used for each of the duplicate membranes. Pre-hybridization and hybridization were carried out according to the manufacturer's instructions (Amersham Biosciences). The membrane was exposed to Hyperfilm ECL (Amersham Biosciences) for visualization.

DNA sequencing and analysis. Each clone was sequenced in duplicate with M13 forward (−20) and/or reverse primers using a BigDye Terminator Cycle Sequencing kit and an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems). Sequence assembly and further editing were performed with CLUSTAL_X (Thompson *et al.*, 1997). The nucleotide sequence identities of the genomic subtracted fragments of *S. pneumoniae* KCTC 5080^T were determined using the BLASTX program available on the BLAST network service of the National Center for Biotechnology Information.

Design of PCR primers and PCR conditions. Oligonucleotide primer sets specific to *S. pneumoniae* were designed using Primer3 software (Rozen & Skaletsky, 2000) based on a 492 bp sequence of the *cpsA* gene from one of the SSH clones. The reaction conditions were optimized using a TGradient Thermal Cycler (Biometra). The *lytA*, *ply*,

Table 1. *Streptococcus* species and other bacteria (*n*=134) used in this study^T, Type strain.

Group	Species	Strain
<i>S. viridans</i> (<i>n</i> =49)	<i>S. pneumoniae</i>	KCTC 5080 ^T , CCARM 4009, CCARM 4019, CCARM 4033, CCARM 4109, CCARM 4112, CCARM 4113, CCARM 4114, CCARM 4115, CCARM 4003, CCARM 4005, CCARM 4078, CCARM 4079, CCARM 4080, CCARM 4084, CCARM 4085, CCARM 4088, CCARM 4106, CCARM 4116, ChDC 4-0134, ChDC 4-0749, ChDC 6-4740, CCARM 4081, CCARM 4091, CCARM 4111, CCARM 4059
	<i>S. pseudopneumoniae</i>	CCUG 49455 ^T , CCUG 48465
	<i>S. mitis</i>	KCTC 3556 ^T , ChDC B183, ChDC B186, ChDC B193, ChDC B194, ChDC B227, ChDC B231, ChDC B239, ChDC B242, ChDC B253, ChDC B258, ChDC B260, ChDC B279, ChDC B286, ChDC B303-1, ChDC B315, ChDC B317
	<i>S. oralis</i>	KCTC 13048 ^T , ATCC 9811, DSM 20379, DSM 20066
Other <i>Streptococcus</i> spp. (<i>n</i> =54)	<i>S. pyogenes</i>	KCTC 3984 ^T , KCTC 3096, KCTC 3208
	<i>S. gordonii</i>	KCTC 3286 ^T , ChDC B180, ChDC B211, ChDC B216, ChDC B241
	<i>S. sanguinis</i>	KCTC 3284 ^T , ChDC B203, ChDC B219, ChDC B304-1, ChDC B304-2, ChDC B304-3, ChDC B305-1, ChDC B305-2, ChDC YSA1
	<i>S. parasanguinis</i>	KCTC 13046 ^T , ChDC B185, ChDC B195, ChDC B212, ChDC B213, ChDC B215, ChDC B217, ChDC OS49
	<i>S. constellatus</i>	ATCC 27823 ^T , ChDC B280, ChDC B284, ChDC B290
	<i>S. intermedius</i>	KCTC 3268 ^T , ChDC KB80, ChDC KB236, ChDC KB717
	<i>S. anginosus</i>	ATCC 33397, ChDC YA1, ChDC YA3, ChDC YA5, ChDC YA6, ChDC YA7, ChDC YA8, ChDC YA9, ChDC YA10, ChDC YA11, ChDC YA12, ChDC YA13, ChDC B181, ChDC YA201, ChDC B232, ChDC B248, ChDC B252, ChDC B263, ChDC B287, ChDC B311, ChDC B407-1
Other coccus species (<i>n</i> =31)	<i>L. plantarum</i>	DSM 20686 ^T
	<i>L. lactis</i> subsp. <i>cremoris</i>	DSM 40699 ^T
	<i>L. lactis</i> subsp. <i>hordniae</i>	KCTC 3768 ^T
	<i>L. piscium</i>	KCTC 3639 ^T
	<i>L. garvieae</i>	KCTC 3772 ^T , LMG 8162, LMG 8501, LMG 9472, LMG 14494
	<i>L. lactis</i> subsp. <i>lactis</i>	KCTC 3769 ^T , KCTC 3191, KCTC 2013, KCTC 3894, KCTC 3926
	<i>E. solitarius</i>	KCTC 3553 ^T
	<i>E. hirae</i>	KCTC 3616 ^T
	<i>E. mundtii</i>	KCTC 3630 ^T
	<i>E. malodoratus</i>	KCTC 3641 ^T
	<i>E. cecorum</i>	KCTC 3642 ^T
	<i>E. saccharolyticus</i>	KCTC 3643 ^T
	<i>E. villorum</i>	KCTC 13904 ^T
	<i>E. haemoperoxidus</i>	KCTC 13910 ^T
	<i>E. moraviensis</i>	KCTC 13911 ^T
	<i>E. phoeniculicola</i>	KCTC 3818 ^T
	<i>E. solitarius</i>	KCTC 3923 ^T
	<i>E. raffinosus</i>	KCTC 5189 ^T
	<i>E. avium</i>	KCTC 5190 ^T
	<i>E. faecalis</i>	KCTC 3206 ^T
	<i>V. fluvialis</i>	LMG 9464 ^T
	<i>V. salmoninarum</i>	LMG 11491 ^T
	<i>V. lutrae</i>	LMG 19537 ^T

Spn9828 and Spn9802 primers were evaluated to compare PCR specificity, as described previously (Suzuki *et al.*, 2005). The PCR was performed with 100 ng genomic DNA as a template in a 20 µl reaction mixture that contained 1 µl each primer, 2 µl 10 × PCR buffer, 1 µl 10 mM dNTPs and 0.3 µl *Taq* DNA polymerase. PCR amplification was carried out using a GeneAmp PCR system 2700 (Applied Biosystems) with the following conditions: for *ply*, Spn9802 and Spn9828 primers, initial denaturation at 94 °C for 2 min and 25 cycles of 94 °C for 10 s,

58 °C for 15 s and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min; for *lytA* primers, initial denaturation at 94 °C for 2 min and 30 cycles of 94 °C for 15 s, 53 °C for 15 s and 72 °C for 15 s, with a final extension at 72 °C for 5 min; for *cpsA* primers, initial denaturation at 94 °C for 5 min and 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 40 s, followed by a final extension at 72 °C for 10 min. The PCR products were analysed in 1.2% Seakem LE agarose gels (FMC Bioproducts) and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Construction and screening of the *S. pneumoniae* SSH library

Approximately 240 putative *S. pneumoniae* KCTC 5080^T clones were obtained from the SSH libraries. To avoid possible false-positive or false-negative clones (Radnedge *et al.*, 2003), all the obtained clones were further screened by reverse Southern dot-blot analysis using two differential probes: one from *S. pneumoniae* KCTC 5080^T and the other from *S. mitis* KCTC 3556^T. A total of 34 *S. pneumoniae* clones were positively identified by the differential probes from *S. pneumoniae* KCTC 5080^T, but were negative using the probes for *S. mitis* KCTC 3556^T. Subsequent nucleotide sequencing demonstrated no redundancy among these 34 selected clones. The low number of identified clones might be due to the high genomic identity between *S. pneumoniae* KCTC 5080^T and *S. mitis* KCTC 3556^T, a closely related viridans group streptococcus.

Identification and annotation of *S. pneumoniae*-specific clones

The identity of the 34 *S. pneumoniae*-specific clones was examined using BLAST homology searches. The resultant data revealed sequences highly homologous to the genomic DNA sequences of *S. pneumoniae* available in GenBank, including four transporter proteins, 10 hypothetical proteins, 14 metabolism proteins, one membrane protein and five miscellaneous proteins.

Among those tested, a single clone possessed the 492 nt sequence specific to *S. pneumoniae* that corresponded to a partial region of the capsular polysaccharide biosynthesis (*cps*) gene. Previously, pneumococcal capsular polysaccharide was determined to be an essential virulence factor of *S. pneumoniae* with anti-phagocytic activity. However, capsule expression can also reduce bacterial attachment to respiratory epithelial cells and inhibit bacterial colonization (Adamou *et al.*, 1998; Cundell *et al.*, 1995; Weiser *et al.*, 1994). Supporting this notion, recent electron microscopy imaging has demonstrated that *S. pneumoniae* cells that are

intimately adherent to epithelial cells have thin capsule layers, indicating downregulation of capsule expression to enhance bacterial adherence (Hammerschmidt *et al.*, 2005). The chromosomal location of the *cps* operon has been localized between the *dexB* and *aliA* genes (García & López, 1997; Guidolin *et al.*, 1994; Morona *et al.*, 1997; Munoz *et al.*, 1997). Although the first four genes (*cpsABCD*) of the *cps* operon are commonly present (García *et al.*, 1999; Jiang *et al.*, 2001), it is known that pneumococcal capsules are structurally distinct among the different serotypes of *S. pneumoniae* (Austrian, 1981; Henrichsen, 1995).

Diagnostic screening of the *cpsA* gene as a novel *S. pneumoniae*-specific marker

To identify genomic markers unique for *S. pneumoniae*, primer sets were designed for the *cpsA* gene (GenBank accession no. NC_011072) (Table 2), and their specificity for *S. pneumoniae* was evaluated with a total of 134 reference strains, including 26 *S. pneumoniae*, two *Streptococcus pseudopneumoniae* and 106 other related cocci, by gradient PCR assay. The PCR results are summarized in Table 3. One of the *cpsA*-specific primer sets (*cpsA*-382F and *cpsA*-735R) was highly specific to *S. pneumoniae* with the expected PCR product of 353 bp, and did not amplify any other reference strain (Fig. 1).

The *lytA*, *ply*, Spn9802 and Spn9828 primers (Table 2) have been used previously for *S. pneumoniae* screening. Because some of the related oral streptococci can harbour the *S. pneumoniae* *lytA* and *ply* genes due to horizontal gene transfer (Seki *et al.*, 2005), a PCR method with Spn9802- and Spn9828-specific primers, in combination with *lytA*- and *ply*-specific primers, has proved useful for identifying *S. pneumoniae*. Although both the Spn9802 and Spn9828 genes encode proteins of unknown function, they are known to be specific to *S. pneumoniae*. Accordingly, both the specificity and efficiency of the *cpsA*-specific primers were compared with those of the primer sets for *lytA*, *ply*, Spn9802 and Spn9828 (Suzuki *et al.*, 2005). Notably, a few false-positive amplifications with the *ply*, Spn9802 and Spn9828 primer sets were observed from the genomic DNAs of *S. mitis* (1/17 strains), *S. pseudopneumoniae* (2/2 strains), *Streptococcus*

Table 2. Oligonucleotide sequences used in this study

Primer	Sequence (5'→3')	Amplicon size (bp)	Reference
<i>cpsA</i> -382F	ACGCAACTGACGAGTGTGAC	353	This study
<i>cpsA</i> -735R	GATCGCGACACCGAACTAAT		
<i>lytA</i> sense	CAACCGTACAGAATGAAGCGG	308	Nagai <i>et al.</i> (2001)
<i>lytA</i> antisense	TTATTCGTGCAATACTCGTGCG		
<i>ply</i> sense	ATTTCTGTAACAGCTACCAACGA	329	Salo <i>et al.</i> (1995)
<i>ply</i> antisense	GAATTCCTGTCTTTTCAAAGTC		
Spn9802-143F	CAAGTCGTTCCAAGGTAACAAGTCT	162	Suzuki <i>et al.</i> (2005)
Spn9802-304R	CTAAACCAACTCGACCACCTCTTT		
Spn9828-19F	GGCATTGTGAATGGATTGATTG	227	Suzuki <i>et al.</i> (2005)
Spn9828-245R	TCATGTGCATCCCAAGCTACA		

Table 3. PCR screening using the primer sets specific for *S. pneumoniae*

Strains: 1, *S. pneumoniae*; 2, *S. mitis*; 3, *S. oralis*; 4, *S. pseudopneumoniae*; 5, *S. pyogenes*; 6, *S. gordonii*; 7, *S. sanguinis*; 8, *S. parasanguinis*; 9, *S. constellatus*; 10, *S. intermedius*; 11, *S. anginosus*; 12, *Lactococcus* spp.; 13, *Enterococcus* spp.; 14, *Vagococcus* spp. +, Positive; –, negative.

Primer set	Result for atypical cocci strains													
	1 (n=26)	2 (n=17)	3 (n=4)	4 (n=2)	5 (n=3)	6 (n=5)	7 (n=9)	8 (n=8)	9 (n=4)	10 (n=4)	11 (n=21)	12 (n=14)	13 (n=14)	14 (n=3)
<i>cpsA</i>	+	+	–	–	–	–	–	–	–	–	–	–	–	–
<i>lytA</i>	+	–	–	–	–	–	–	–	–	–	–	–	–	–
<i>ply</i>	+	+	–	–	–	+	+	–	–	–	–	–	+	–
Spn9802	+	+	–	–	–	+	+	–	–	–	–	–	–	–
Spn9828	+	+	–	+	–	–	–	–	–	–	–	–	–	–
Subtotal	25	1	16	1	4	1	1	3	3	2	7	2	8	4

Fig. 1. Electrophoretic examination of *S. pneumoniae*-specific PCR products generated with primers *cpsA*-382F and *cpsA*-735R based on the *cpsA* gene. Lanes: M, GeneRuler 1 kb DNA ladder (Fermentas); 1, *S. pneumoniae* KCTC 5080^T; 2, *S. pneumoniae* CCARM 4009; 3, *S. pneumoniae* CCARM 4019; 4, *S. pneumoniae* CCARM 4033; 5, *S. pneumoniae* CCARM 4109; 6, *S. pneumoniae* CCARM 4112; 7, *S. pneumoniae* CCARM 4113; 8, *S. pneumoniae* CCARM 4114; 9, *S. pneumoniae* CCARM 4115; 10, *S. pneumoniae* CCARM 4003; 11, *S. pneumoniae* CCARM 4005; 12, *S. pneumoniae* CCARM 4078; 13, *S. pneumoniae* CCARM 4079; 14, *S. pneumoniae* CCARM 4080; 15, *S. pneumoniae* CCARM 4080; 16, *S. pneumoniae* CCARM 4085; 17, *S. pneumoniae* CCARM 4088; 18, *S. pneumoniae* CCARM 4106; 19, *S. pneumoniae* CCARM 4116; 20, *S. pneumoniae* ChDC 4-0134; 21, *S. pneumoniae* ChDC 4-0749; 22, *S. pneumoniae* ChDC 6-4740; 23, *S. pneumoniae* CCARM 4081; 24, *S. pneumoniae* CCARM 4091; 25, *S. pneumoniae* CCARM 4111; 26, *S. pneumoniae* CCARM 4059; 27, *S. pseudopneumoniae* CCUG 49455^T; 28, *S. pseudopneumoniae* CCUG 48465; 29, *S. mitis* KCTC 3556^T; 30, *S. mitis* ChDC B183; 31, *S. mitis* ChDC B186; 32, *S. mitis* ChDC B193; 33, *S. mitis* ChDC B194; 34, *S. mitis* ChDC B227; 35, *S. mitis* ChDC B231; 36, *S. mitis* ChDC B239; 37, *S. mitis* ChDC B242; 38, *S. mitis* ChDC B253; 39, *S. mitis* ChDC B258; 40, *S. mitis* ChDC B260; 41, *S. mitis* ChDC B279; 42, *S. mitis* ChDC B286; 43, *S. mitis* ChDC B303-1; 44, *S. mitis* ChDC B315; 45, *S. mitis* ChDC B317; 46, *S. oralis* KCTC 13048^T; 47, *S. oralis* ATCC 9811; 48, *S. oralis* DSM 20379. Other *Streptococcus* and other bacterial (*Lactococcus* or *Enterococcus*) species are not shown because the results for these strains are the same as those seen in lanes 27–48; no *S. pneumoniae*-specific DNA is present. The arrow shows the expected PCR product of 353 bp common to all *S. pneumoniae* strains (lanes 1–26).

gordonii (1/5 strains) and *Streptococcus sanguinis*, whilst one *S. pneumoniae* (1/26 strains) did not amplify with the *lytA* primers. In addition, 10 out of 21 *Streptococcus anginosus* strains produced amplicons of ~800 bp using the Spn9828 primers, although they should have yielded amplified products of 227 bp. The false-positive results gave amplified PCR products from unexpected species or with different sizes. This was mainly caused by non-specific primers but sometimes also by a low annealing temperature. The results indicated that these previously described *S. pneumoniae*-specific primers can produce both false-positive and false-negative results.

S. pseudopneumoniae is very closely related to *S. pneumoniae*. Pairwise comparisons show that their 16S rRNA gene sequences are almost identical, with a difference of only 5 bp between the two species, corresponding to 99.7 % identity. Consistent with this finding, a recent study demonstrated that the Spn9802 and *ply* primer sets could not differentiate *S. pneumoniae* and the recently acknowledged species *S. pseudopneumoniae* (Abdeldaim *et al.*, 2008). Surprisingly, the current *cpsA*-specific primer set differentiated *S. pneumoniae* from *S. pseudopneumoniae*. Thus, this primer set may be useful for discrimination of both species, as it is well known that *S. pseudopneumoniae* strains have no pneumococcal capsules, unlike *S. pneumoniae* (Keith *et al.*, 2006).

Recently, several real-time PCR assays have been developed for *S. pneumoniae* (Abdeldaim *et al.*, 2008; Carvalho *et al.*, 2007; Corless *et al.*, 2001; Greiner *et al.*, 2001; McAvin *et al.*, 2001; Saukkoripi *et al.*, 2004). Although these methods are sensitive, the results depend on the uniqueness of the applied probes. Among these, the most recent Spn9802-based real-time PCR assay gave positive results with *S. pseudopneumoniae* strains (CCUG 49455^T and CCUG 48465) and *S. pneumoniae* (Abdeldaim *et al.*, 2008). PCR with the *cpsA*-specific primer set efficiently detected genomic DNA purified from all 26 *S. pneumoniae* strains, and no other reference strain including two *S. pseudopneumoniae* strains was amplified. Therefore, the *cpsA* gene could be used as a new marker for detection of *S. pneumoniae* using a real-time detection assay.

Because *S. pneumoniae* shares over 99 % 16S rRNA gene sequence identity with the other viridans group streptococci and because lateral gene transfer occurs among these species, the identification of novel genetic markers specific for *S. pneumoniae* is needed for accurate diagnosis and discrimination. Our findings suggest that the *cpsA*-specific primer set can discriminate *S. pneumoniae* from the closely related viridans group streptococci as well as other pneumococcus-like streptococci such as *S. pseudopneumoniae*. Moreover, the *cpsA*-specific primer set yielded maximal amplification at temperatures ranging from 53 to 63 °C as well as enhanced sensitivity compared with the previous Spn9802- and Spn9828-specific primers, implying a potential for novel multiplex or real-time PCR for the specific detection of *S. pneumoniae*.

In this study, SSH was used to identify the *cpsA* gene as a novel genomic marker specific for *S. pneumoniae*. The *cpsA*-specific primer set discriminated *S. pneumoniae* from other closely related viridans group members. This new primer set may be useful for the routine diagnosis and identification of presumptive *S. pneumoniae* isolates. Alternatively, the *cpsA* gene can be used as an additional genomic marker specific for *S. pneumoniae* in combination with previously described genetic markers such as *lytA*, *ply*, Spn9802 and Spn9828.

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REFERENCES

- Abdeldaim, G. M., Stralin, K., Olcen, P., Blomberg, J. & Herrmann, B. (2008). Toward a quantitative DNA-based definition of pneumococcal pneumonia: a comparison of *Streptococcus pneumoniae* target genes, with special reference to the Spn9802 fragment. *Diagn Microbiol Infect Dis* **60**, 143–150.
- Adamou, J. E., Wizemann, T. M., Barren, P. & Langermann, S. (1998). Adherence of *Streptococcus pneumoniae* to human bronchial epithelial cells (BEAS-2B). *Infect Immun* **66**, 820–822.
- Agron, P. G., Macht, M., Radnedge, L., Skowronski, E. W., Miller, W. & Andersen, G. L. (2002). Use of subtractive hybridization for comprehensive surveys of prokaryotic genome differences. *FEMS Microbiol Lett* **211**, 175–182.
- Akopyants, N. S., Fradkov, A., Diatchenko, L., Hill, J. E., Siebert, P. D., Lukyanov, S. A., Sverdlov, E. D. & Berg, D. E. (1998). PCR-based subtractive hybridization and differences in gene content among strains of *Helicobacter pylori*. *Proc Natl Acad Sci U S A* **95**, 13108–13113.
- Austrian, R. (1981). Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. *Rev Infect Dis* **3**, S1–S17.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. & Struhl, K. (1993). *Current Protocols in Molecular Biology*, Section 2.4.1–2.4.2. New York: John Wiley & Sons.
- Carvalho, M. da G. S., Tondella, M. L., McCaustland, K., Weidlich, L., McGee, L., Mayer, L. W., Steigerwalt, A., Whaley, M., Facklam, R. R. & other authors (2007). Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. *J Clin Microbiol* **45**, 2460–2466.
- Corless, C. E., Guiver, M., Borrow, R., Edwards-Jones, V., Fox, A. J. & Kaczmarek, E. B. (2001). Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J Clin Microbiol* **39**, 1553–1558.
- Cundell, D. R., Weiser, J. N., Shen, J., Young, A. & Tuomanen, E. I. (1995). Relationship between colonial morphology and adherence of *Streptococcus pneumoniae*. *Infect Immun* **63**, 757–761.
- Dyson, C., Barnes, R. A. & Harrison, G. A. (1999). Infective endocarditis: an epidemiological review of 128 episodes. *J Infect* **38**, 87–93.
- Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P., Jr (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

- García, E. & López, R. (1997). Molecular biology of the capsular genes of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* **149**, 1–10.
- García, E., Llull, D. & Lopez, R. (1999). Functional organization of the gene cluster involved in the synthesis of the pneumococcal capsule. *Int Microbiol* **2**, 169–176.
- Greiner, O., Day, P. J., Bosshard, P. P., Imeri, F., Altwegg, M. & Nadal, D. (2001). Quantitative detection of *Streptococcus pneumoniae* in nasopharyngeal secretions by real-time PCR. *J Clin Microbiol* **39**, 3129–3134.
- Guidolin, A., Morona, J. K., Morona, R., Hansman, D. & Paton, J. C. (1994). Nucleotide sequence analysis of genes essential for capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* type 19F. *Infect Immun* **62**, 5384–5396.
- Hammerschmidt, S., Wolff, S., Hocke, A., Rosseau, S., Muller, E. & Rohde, M. (2005). Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infect Immun* **73**, 4653–4667.
- Henrichsen, J. (1995). Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol* **33**, 2759–2762.
- Jiang, S. M., Wang, L. & Reeves, P. R. (2001). Molecular characterization of *Streptococcus pneumoniae* type 4, 6B, 8, and 18C capsular polysaccharide gene clusters. *Infect Immun* **69**, 1244–1255.
- Kawamura, Y., Whiley, R. A., Shu, S. E., Ezaki, T. & Hardie, J. M. (1999). Genetic approaches to the identification of the mitis group within the genus *Streptococcus*. *Microbiology* **145**, 2605–2613.
- Keith, E. R., Podmore, R. G., Anderson, T. P. & Murdoch, D. R. (2006). Characteristics of *Streptococcus pseudopneumoniae* isolated from purulent sputum samples. *J Clin Microbiol* **44**, 923–927.
- Kim, W., Kim, J.-Y., Cho, S.-L., Nam, S.-W., Shin, J.-W., Kim, Y.-S. & Shin, H.-S. (2008). Glycosyltransferase – a specific marker for the discrimination of *Bacillus anthracis* from the *Bacillus cereus* group. *J Med Microbiol* **57**, 279–286.
- McAvin, J. C., Reilly, P. A., Roudabush, R. M., Barnes, W. J., Salmen, A., Jackson, G. W., Beninga, K. K., Astorga, A., McCleskey, F. K. & other authors (2001). Sensitive and specific method for rapid identification of *Streptococcus pneumoniae* using real-time fluorescence PCR. *J Clin Microbiol* **39**, 3446–3451.
- Morona, J. K., Morona, R. & Paton, J. C. (1997). Characterization of the locus encoding the *Streptococcus pneumoniae* type 19F capsular polysaccharide biosynthetic pathway. *Mol Microbiol* **23**, 751–763.
- Morrison, K. E., Lake, D., Crook, J., Carlone, G. M., Ades, E., Facklam, R. & Sampson, J. S. (2000). Confirmation of *psaA* in all 90 serotypes of *Streptococcus pneumoniae* by PCR and potential of this assay for identification and diagnosis. *J Clin Microbiol* **38**, 434–437.
- Munoz, R., Mollerach, M., Lopez, R. & García, E. (1997). Molecular organization of the genes required for the synthesis of type 1 capsular polysaccharide of *Streptococcus pneumoniae*: formation of binary encapsulated pneumococci and identification of cryptic dTDP-rhamnose biosynthesis genes. *Mol Microbiol* **25**, 79–92.
- Nagai, K., Shibasaki, Y., Hasegawa, K., Davies, T. A., Jacobs, M. R., Ubukata, K. & Appelbaum, P. C. (2001). Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and β -lactam resistance, and to detect common macrolide resistance determinants. *J Antimicrob Chemother* **48**, 915–918.
- O'Neill, A. M., Gillespie, S. H. & Whiting, G. C. (1999). Detection of penicillin susceptibility in *Streptococcus pneumoniae* by *pbp2b* PCR-restriction fragment length polymorphism analysis. *J Clin Microbiol* **37**, 157–160.
- Radnedge, L., Agron, P. G., Hill, K. K., Jackson, P. J., Ticknor, L. O., Keim, P. & Andersen, G. L. (2003). Genome differences that distinguish *Bacillus anthracis* from *Bacillus cereus* and *Bacillus thuringiensis*. *Appl Environ Microbiol* **69**, 2755–2764.
- Rozen, S. & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365–386.
- Salo, P., Ortvist, A. & Leinonen, M. (1995). Diagnosis of bacteremic pneumococcal pneumonia by amplification of pneumolysin gene fragment in serum. *J Infect Dis* **171**, 479–482.
- Saukkoriipi, A., Leskela, K., Herva, E. & Leinonen, M. (2004). *Streptococcus pneumoniae* in nasopharyngeal secretions of healthy children: comparison of real-time PCR and culture from STGG-transport medium. *Mol Cell Probes* **18**, 147–153.
- Seki, M., Yamashita, Y., Torigoe, H., Tsuda, H., Sato, S. & Maeno, M. (2005). Loop-mediated isothermal amplification method targeting the *lytA* gene for detection of *Streptococcus pneumoniae*. *J Clin Microbiol* **43**, 1581–1586.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Stackebrandt, E., Witt, D., Kemmerling, C., Kroppenstedt, R. & Liesack, W. (1991). Designation of streptomycete 16S and 23S rRNA-based target regions for oligonucleotide probes. *Appl Environ Microbiol* **57**, 1468–1477.
- Suzuki, N., Seki, M., Nakano, Y., Kiyoura, Y., Maeno, M. & Yamashita, Y. (2005). Discrimination of *Streptococcus pneumoniae* from viridans group streptococci by genomic subtractive hybridization. *J Clin Microbiol* **43**, 4528–4534.
- Suzuki, N., Yuyama, M., Maeda, S., Ogawa, H., Mashiko, K. & Kiyoura, Y. (2006). Genotypic identification of presumptive *Streptococcus pneumoniae* by PCR using four genes highly specific for *S. pneumoniae*. *J Med Microbiol* **55**, 709–714.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Verhelst, R., Kaijalainen, T., De Baere, T., Verschraegen, G., Claeys, G., Van Simaey, L., De Ganck, C. & Vanechoutte, M. (2003). Comparison of five genotypic techniques for identification of optochin-resistant pneumococcus-like isolates. *J Clin Microbiol* **41**, 3521–3525.
- Weiser, J. N., Austrian, R., Sreenivasan, P. K. & Masure, H. R. (1994). Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infect Immun* **62**, 2582–2589.
- Whatmore, A. M., Efstratiou, A., Pickerill, A. P., Broughton, K., Woodard, G., Sturgeon, D., George, R. & Dowson, C. G. (2000). Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: characterization of “atypical” pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor-encoding genes. *Infect Immun* **68**, 1374–1382.
- Willcox, M. D., Drucker, D. B. & Hillier, V. F. (1988). In-vitro adherence of oral streptococci in the presence of sucrose and its relationship to cariogenicity in the rat. *Arch Oral Biol* **33**, 109–113.