ORIGINAL ARTICLE BACTERIOLOGY

Development of a real-time PCR assay for the specific detection and identification of *Streptococcus pseudopneumoniae* using the *recA* gene

V. Sistek[1,2], M. Boissinot[1,2], D. K. Boudreau[1], A. Huletsky[1,2], F. J. Picard[1] and M. G. Bergeron[1,2]

1) *Centre de recherche en infectiologie de l'Université Laval, Centre Hospitalier Universitaire de Québec (Pavillon CHUL) and* 2) *Département de microbiologie-infectiologie et d'immunologie, Faculté de médecine, Université Laval, Québec, QC, Canada*

Abstract

We sequenced the evolutionarily conserved genes 16S rRNA, *atpD*, *tuf,* and *recA* from *Streptococcus pseudopneumoniae*, *Streptococcus pneumoniae*, *Streptococcus mitis*, and *Streptococcus oralis*. Phylogenetic analysis revealed that *recA* provided good resolution between these species, including discrimination of the novel species *S. pseudopneumoniae*. By contrast, the more conserved 16S rRNA, *tuf* and *atpD* are not sufficiently discriminatory. Therefore, *recA* sequences were used to develop a real-time PCR assay with a locked nucleic acid-mediated TaqMan probe for the specific detection and identification of *S. pseudopneumoniae*. The PCR assay showed excellent specificity and a detection limit of <10 genome copies for the detection and identification of *S. pseudopneumoniae* strains, which makes it a promising tool for molecular identification and epidemiological studies. In conclusion, this article describes for the first time a PCR assay for the specific identification of *S. pseudopneumoniae*.

Keywords: DNA sequencing, genomic identification, real-time PCR, *recA* gene, *Streptococcus pseudopneumoniae*
Original Submission: 15 June 2010; **Revised Submission:** 14 October 2010; **Accepted:** 25 October 2010
Editor: M. Drancourt
Article published online: 25 October 2011
Clin Microbiol Infect 2012; **18:** 1089–1096
10.1111/j.1469-0691.2011.03684.x

Corresponding author: M. G. Bergeron, Centre de recherche en infectiologie de l'Université Laval, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, 2705 Boul. Laurier, Québec G1V 4G2, Canada
E-mail: michel.g.bergeron@crchul.ulaval.ca

Introduction

Streptococcus pseudopneumoniae is a novel species related to the mitis group of streptococci, which includes 15 species [1]. Among these species, *S. pseudopneumoniae* is phenotypically and genetically close to *Streptococcus pneumoniae*, *Streptococcus mitis*, and *Streptococcus oralis* [2,3]. All *S. pseudopneumoniae* strains described to date have been isolated from lower respiratory tract samples [2,4–7]. The clinical importance of this species is currently unknown [6,7]. However, Keith *et al.* [7] observed that patients with *S. pseudopneumoniae* infection were more likely to have a history of chronic obstructive pulmonary disease or exacerbation of chronic obstructive pulmonary disease. Moreover, antibiotic-resistant strains of *S. pseudopneumoniae* have already been described [5,7–9].

Currently, *S. pseudopneumoniae* can be identified on the basis of bile solubility and optochin tests. *S. pseudopneumoniae* cells are not soluble in bile, and are resistant or intermediate to optochin when incubated under an atmosphere supplemented with 5% CO_2, but are susceptible to optochin when incubated in air [2]. Various commercial phenotype-based tests have been evaluated, but these tests fail to differentiate *S. pseudopneumoniae* and *S. pneumoniae* [2]. PCR assays targeting 16S rRNA, pneumococcal surface adhesion (*psaA*), pneumolysin (*ply*), D-alanine:D-alanine ligase (*ddl*), glutamate dehydrogenase (*gdh*), the β-subunit of RNA polymerase (*rpoB*) and manganese-dependent superoxide dismutase (*sodA*) gene sequences have been published as genetic methods for the identification of pneumococci. However, none of these molecular tests can discriminate *S. pseudopneumoniae* from *S. pneumoniae* [2,4]. PCR assays based on the autolysin gene (*lytA*) have shown that *S. pneumoniae* can be discriminated from pneumococcus-like viridans group streptococci, including *S. pseudopneumoniae*. However, the use of *lytA* as a molecular

target does not enable *S. pseudopneumoniae* to be distinguished from other viridans streptococci [4,10].

The increasing number of newly identified or previously unrecognized pathogens, the availability of new antimicrobial agents and the evolution of bacterial resistance mechanisms have contributed to changes in the epidemiology and treatment of respiratory tract infections. The development of new diagnostic tests for pathogen identification and determination of resistance profiles will certainly improve the selection of appropriate antibiotic therapy for the treatment of lower respiratory tract infections [11]. Consequently, it is important for the laboratory to differentiate *S. pneumoniae*, *S. pseudopneumoniae,* and the mitis group streptococci, notably *S. mitis* and *S. oralis*, as identification may influence diagnosis and treatment.

In this study, we analysed four conserved genes—16S rRNA, *atpD* (encoding the F_0F_1 H^+-ATPase β-subunit), *tuf* (encoding elongation factor Tu), and *recA* (encoding RecA, a protein contributing to homologous recombination)—that have already been used to reconstruct bacterial phylogeny [13–16]. Their capacity for species discrimination was evaluated. We also developed a real-time PCR assay for the detection and identification of *S. pseudopneumoniae*.

Materials and Methods

Strains

A complete list of strains used in this study is provided in Table S1. All clinical strains, from the Centre de Recherche en Infectiologie, were identified phenotypically with the Positive Breakpoint Combo 20 of the Microscan system (Dade Behring, West Sacramento, CA, USA). Isolates were cultured on sheep blood agar plates at 35°C under a 5% CO_2-enriched atmosphere unless otherwise stated.

TABLE 1. Phenotypic test results for *Streptococcus pseudopneumoniae* and closely related isolates

Species[a]	Strain no.[b]	Optochin (in O_2)[c]	Optochin (with 5% CO_2)[c]	Bile solubility[d]
S. pneumoniae	ATCC 51916	S	S	S
S. pneumoniae	ATCC 700673	S	S	S
S. pneumoniae	CCRI-1393	S	S	S
S. pneumoniae	CCRI-17887	S	S	S
S. pneumoniae	CCRI-18437	S	S	S
S. pneumoniae	CCRI-1396	S	S	S
S. pneumoniae[e]	CCRI-8999	S	S	S
S. pneumoniae[e]	CCRI-14740	S	S	S
S. pneumoniae[e]	CCRI-14753	S	S	S
S. pneumoniae[e]	CCRI-14763	S	S	S
S. pneumoniae[e]	CCRI-15209	S	S	S
S. pneumoniae[e]	CCRI-15786	S	S	S
S. pneumoniae[e]	CCRI-17733	S	S	S
S. pneumoniae[e]	CCRI-15796	S	S	S
S. pneumoniae[e]	578	S	S	I
S. pneumoniae[e]	1504	S	S	I
S. pneumoniae[e]	101/87	R	R	I
S. pseudopneumoniae[f]	CCRI-8984	S	R	I
S. pseudopneumoniae[f]	CCRI-14743	S	R	I
S. pseudopneumoniae[f]	CCRI-18123	S	R	I
S. pseudopneumoniae	CCUG 49455^T	S	R	I
S. pseudopneumoniae	CCUG 50868	S	R	I
S. pseudopneumoniae	CCUG 50869	S	R	I
S. pseudopneumoniae	CCUG 50870	S	R	I
S. pseudopneumoniae	2482-91	S	R	I
S. pseudopneumoniae	2483-91	S	R	I
S. pseudopneumoniae	2497-91	S	R	I
S. pseudopneumoniae	2987-98	S	R	I
S. mitis	ATCC 49456^T	R	R	I
S. mitis	CCRI-1398	R	R	I
S. mitis	CCRI-14944	R	R	I
S. mitis	CCRI-14955	R	R	I
S. mitis	CCRI-15019	R	R	I
S. mitis	CCRI-15034	R	R	I
S. mitis	CCRI-15059	R	R	I
S. mitis	CCRI-17399	R	R	I
S. oralis	ATCC 35037^T	R	R	I
S. oralis	ATCC 10557	R	R	I
S. oralis	CCRI-15017	R	R	I
S. oralis	CCRI-15027	R	R	I

[a]Identification by optochin test, bile solubility, and genotyping.
[b] ^{T} indicates type strain.
[c]Optochin test: S, susceptible; R, resistant.
[d]Bile solubility: S, soluble; I, insoluble.
[e]*S. pneumoniae* atypical.
[f]Formerly *S. pneumoniae*.

Phenotypic identification

Phenotypes of all *S. pseudopneumoniae* isolates, and some isolates of *S. pneumoniae*, *S. mitis*, and *S. oralis*, were confirmed by optochin sensitivity and bile solubility as previously described [2] (Table 1).

Sequencing

PCR amplifications were performed with crude DNA extracts, using the primers described in Table 2. Crude DNA extracts from bacteria were prepared by using the BD GeneOhm Lysis Kit (BD Diagnostics-GeneOhm, Quebec, QC, Canada), as recommended by the manufacturer. PCR amplifications, sequencing and phylogenetic analysis were performed as previously described [14]. The GenBank accession numbers of 16S rRNA, *atpD*, *tuf*, and *recA* sequences are shown in Figs 1 and 2, and Fig. S1.

Real-time PCR assay

A 135-bp fragment of *recA* gene was amplified by use of the following reaction mixture: 0.05 U/μL *Taq* DNA polymerase (Promega, Madison, WI, USA) combined with the TaqStart Antibody (Clontech Takara Bio, Mountain View, CA, USA), 200 μM dNTP (Amersham Biosciences, Piscataway, NJ, USA), 10 mM Tris-HCl (pH 9.1), 50 mM KCl, 3.3 mg/mL bovine serum albumin (Sigma-Aldrich Canada, Oakville, Ontario, Canada), 0.1% Triton, 2.5 mM $MgCl_2$, 0.6 μM primers, and 0.2 μM probe (Table 2). One microlitre of sample DNA was added to the reaction mixture to give a final 25-μL reaction volume. DNA was amplified with a Rotor-Gene 3000 (Corbett Research, Bath, UK) by using the following cycling parameters: an initial denaturing step at 94°C for 3 min, followed by 45 cycles of a denaturing step at 95°C for 5 s, an annealing step at 57°C for 15 s, and an extension step at 72°C for 30 s. Data acquisition and analysis for the real-time PCR assay were performed with the Rotor-Gene data analysis software (Corbett Research supporting programs).

Purification of genomic DNA

Colonies of pure cultures were treated with the BD GeneOhm Lysis Kit (BD Diagnostics-GeneOhm), as recommended by the manufacturer. The crude DNA extracts were then treated with RNase (Qiagen, Mississauga, Ontario, Canada) for 15 min at 55°C. Genomic DNA from the crude extracts was purified with the MagneSil KF Genomic System (Promega) on a KingFisher instrument (ThermoLabsystem, Dreieich, Germany).

Specificity and sensitivity of the real-time PCR

Specificity tests were performed with 1 ng/μL purified genomic DNA from 11 *S pseudopneumoniae* isolates, 12 *S. pneumoniae* isolates, six *S. mitis* isolates, and four *S. oralis* isolates (Table 1). Four *S. pseudopneumoniae* strains (CCUG 49455^T, CCUG 50869, CCUG 50868, and CCRI-8984) were used to determine the sensitivity of the real-time PCR assay. Serial ten-fold dilutions (ranging from 10^5 to 10 copies) of purified genomic DNA from these strains were used for sensitivity tests.

Results and Discussion

Evaluation of 16S rRNA, *atpD*, *tuf*, and *recA gene* for *S. pseudopneumoniae* identification

Initially, the four conserved genes were sequenced from 22 type and reference strains of *S. pseudopneumoniae* (n = 4),

TABLE 2. Primers and probe used for PCR amplification/sequencing and real-time PCR

Target gene	Primer/probe name	Primer/probe sequence[a]	PCR annealing temperature (°C)	Expected size (bp)
PCR amplification/sequencing				
recA	RStrGseq81	GAAAWWIATYGARAAAGAITTTGGTAA	54	852
	RStrGseq937	TTYTCAGAWCCTTGICCAATYTTYTC		
tuf	UTseq271	AAYATGATIACIGGIGCIGCICARATGGA	57	881
	UTseq1138	CCIACIGTICKICCRCCYTCRCG		
atpD	UAseq27b	RTIRYIGGICCIGTIRTIGAYGT	54	902
	UAseq883	TCRTCIGCIGGIACRTAIAYIGCYTG		
16S rDNA	SSU27	AGAGTTTGATCMTGGCTCAG	58	1510
	SSU536[b]	GTGCCAGCMGCCGCGGTAATAC		
	SSU685[b]	TCTACGCATTTCACYGCTAC		
	SSU926[b]	AAACTYAAAKGAATTGACGG		
	SSU1492	TACGGYTACCTTGTTACGACTT		
Real-time PCR				
recA	RSpneSpse677	GTGCTITGAAATTCTATGCTTC	57	135
	RSpneSpse790	GTGGAGCTACCTTATTTTTTAC		
	RSpse-TL1-B1[c,d,e]	TET-TGGTA**ACA**CA**C**AAATTAAG-BHQ1		

[a]I, inosine; Y, C or T; R, A or G; K, G or T; M, A or C; W, A or T.
[b]Internal primers used for sequencing only.
[c]Bold and underlined nucleotides represent LNA residues.
[d]TET, 6-tetracholorofluorescein.
[e]BHQ1, black hole quencher.

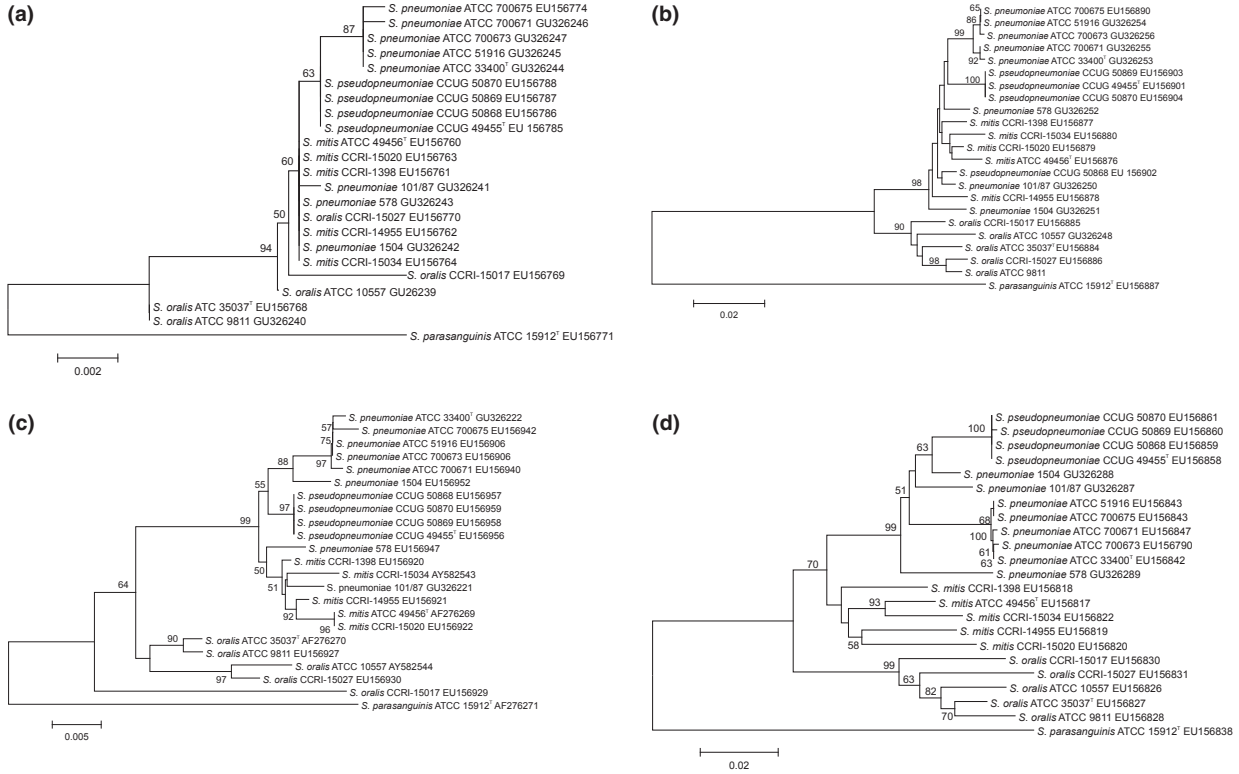

FIG. 1. Phylogenetic trees of type and reference strains based on sequences of four housekeeping genes: (a) 16S rRNA, (b) *atpD*, (c) *tuf*, and (d) *recA*. The distance trees were constructed with the neighbour-joining method and Kimura's two-parameter substitution model. The value on each branch is the estimated confidence limit (expressed as a percentage) for the position of the branch as determined by bootstrap analysis (1000 random resamplings). Only values exceeding 50% are shown. *Streptococcus parasanguinis* was used as outgroup. The scale bar represents a percentage evolutionary distance. ^{T} indicates species type strains.

S. pneumoniae (n = 8), *S. mitis* (n = 5), and *S. oralis* (n = 5). The sequences were analysed, and phylogenetic trees were constructed for each gene to discriminate *S. pseudopneumoniae* at the species level. The tree topologies obtained with the neighbour-joining method were evaluated and confirmed by maximum-parsimony analysis (data not shown). Among the *S. pneumoniae* strains used, three were considered to be 'atypical' *S. pneumoniae* strains (101/87, 578, and 1504, originally characterized by Diaz *et al.* [3]). The 'atypical' character of *S. pneumoniae* strains is defined as aberrant reactions to optochin susceptibility and/or deoxycholate (bile) solubility. Atypical pneumococcal isolates are also genetically distinct from, although closely related to, typical pneumococci. Indeed, Whatmore *et al.* [12] described strain 101/87 as the prototype of atypical *S. pneumoniae*.

16S rDNA sequencing is well established as a standard method for the identification of bacterial species [17,18]. To verify its capacity for discrimination of *S. pseudopneumoniae*, sequences covering a 1471-bp segment of 16S rDNA from the streptococcal strains were analysed. The 16S rDNA sequences showed a high degree of homology within the members of the mitis group, and minor differences in nucleotide sequences (≤0.5%) between *S. pseudopneumoniae* and *S. pneumoniae*. The 16S rRNA-based tree also revealed poor resolution between *S. pseudopneumoniae* and *S. pneumoniae* species (Fig. 1a). Moreover, the *S. pseudopneumoniae* strain cluster was not well supported by the bootstrap value at the node (63%). These results are in agreement with the results previously described by Arbique *et al.* [2]. The high degree of identity of 16S rDNA sequences between *S. pseudopneumoniae* and *S. pneumoniae* precluded distinction between these species. Although sequencing of 16S r2DNA represents an excellent tool for the identification of most organisms, it often has insufficient discriminatory power to distinguish closely related species, such as members of the mitis group of streptococci [2].

Use of *atpD* gene has previously been described for the identification of *Enterobacteriaceae* [14]. In the present study, analysis of a 787-bp segment of *atpD* did not permit discrimination of *S. pseudopneumoniae* from *S. pneumoniae* and *S. mitis*. The *atpD*-based tree revealed a main cluster formed by typical *S. pneumoniae* strains, whereas it did not show a

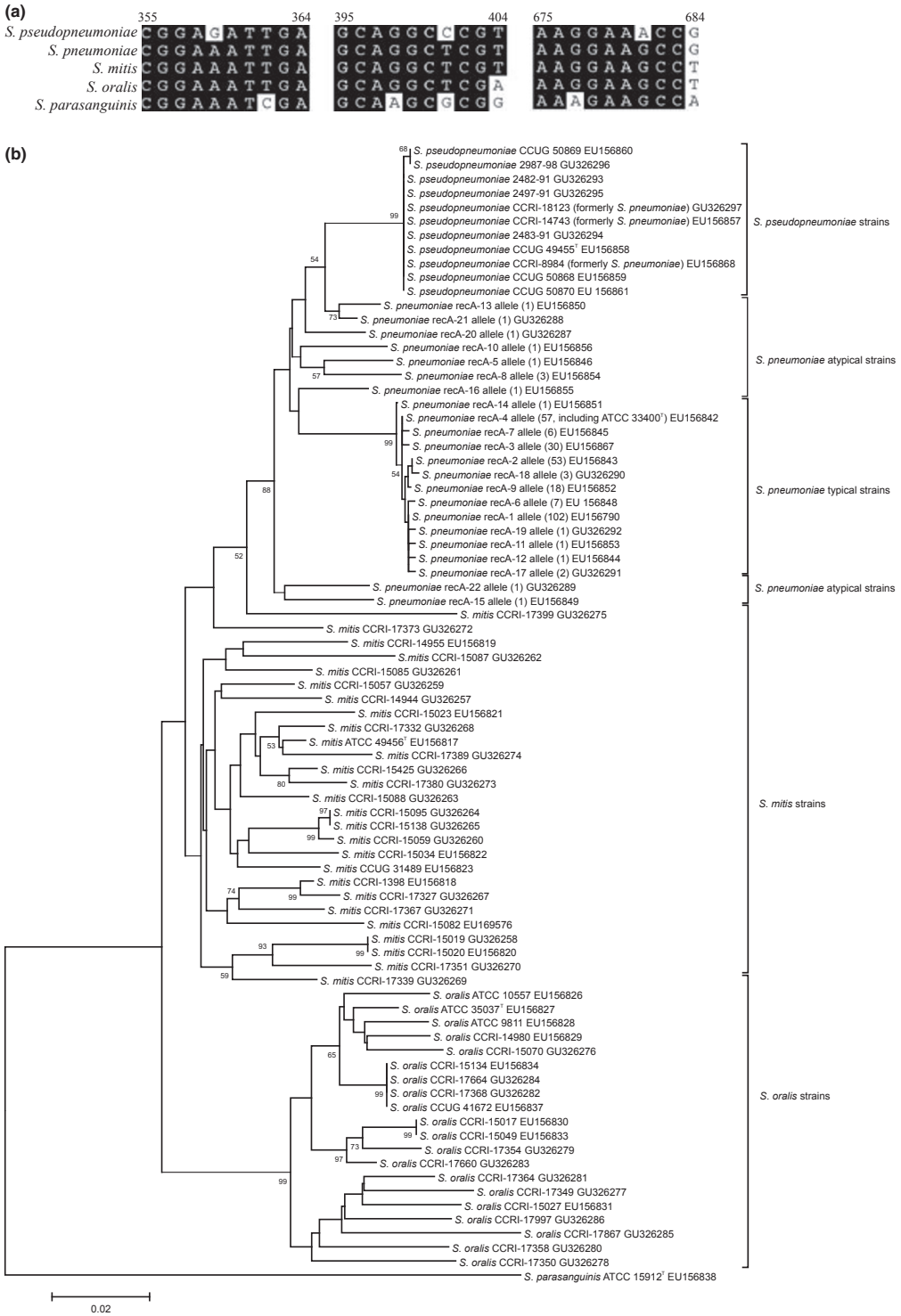

FIG. 2. (a) Nucleotide sequence signature of *Streptococcus pseudopneumoniae* species. Numbers above the sequence alignment indicate nucleotide positions. (b) Phylogenetic tree of clinical isolates based on *recA* gene sequences. The distance tree was constructed with the neighbour-joining method and Kimura's two-parameter substitution model. The value on each branch is the estimated confidence limit (expressed as a percentage) for the position of the branch as determined by bootstrap analysis (1000 random resamplings). Only values exceeding 50% are shown. *Streptococcus parasanguinis* was used as outgroup. The scale bar represents a 2% evolutionary distance. Genbank accession numbers are provided for each allele of *Streptococcus pneumoniae* and for each strain of *S. pseudopneumoniae*, *Streptococcus mitis*, and *Streptococcus oralis*. Numbers in parenthesis indicate total of strains per *S. pneumoniae* allele. ^{T} indicates species type strains.

clear separation between *S. pseudopneumoniae*, atypical *S. pneumoniae*, and *S. mitis* (Fig. 1b). This may reflect a complex evolutionary picture for *atpD* gene. Balsalobre *et al.* [19] showed that the optochin susceptibility of some isolates of the mitis group was attributable to the acquisition of *atpC*, *atpA* and part of *atpB* by horizontal gene transfer. Horizontal gene transfer or other recombination events could have also affected the ATP synthase operon in the *atpD* region, thus blurring the evolutionary signal between *S. pseudopneumoniae*, *S. pneumoniae*, and *S. mitis*.

In our previous study, sequence analysis of *tuf* from 28 streptococcal species (including *S. pneumoniae*, *S. mitis*, and *S. oralis*) proved this gene to be suitable for phylogenetic analysis of streptococci [15]. In the present study, *S. pseudopneumoniae* was added to the analysis. The phylogenetic tree (based on sequences covering a 787-bp segment of *tuf*) revealed that the four *S. pseudopneumoniae* strains grouped together (bootstrap value of 97%) and had identical nucleotide sequences (including the type strain) (Fig. 1c). Consequently, *tuf* sequence phylogeny appears to be suitable for the identification of *S. pseudopneumoniae*. Several studies have demonstrated the usefulness of *recA* sequences for the differentiation of species and subspecies within various bacterial genera, such as mycobacteria, vibrios, and lactobacilli [16,20–23]. Analysis of an 817-bp segment of *recA* showed that sequences of *S. pseudopneumoniae* were highly conserved. The *recA*-based tree showed that *S. pseudopneumoniae* strains clustered with a bootstrap value of 100% at the node (Fig. 1d). Moreover, a multiple sequence alignment revealed three nucleotide variations shared by all *S. pseudopneumoniae* strains but not found in other members of the mitis group (Fig. 2a). One of these nucleotide variations resulted in an amino acid change (Thr275 → Ala) corresponding to a unique protein signature common to all *S. pseudopneumoniae* strains. Therefore, the *recA* sequences could constitute a good tool for identification of *S. pseudopneumoniae*.

Distinction of clinical isolates by *tuf* and *recA* sequencing

In order to validate the use of *tuf* and *recA* sequences for identification of *S. pseudopneumoniae*, partial sequences of these two genes were determined for 334 isolates of the mitis group (see Table S1), and phylogenetic trees were constructed with the neighbour-joining method. In order to simplify the phylogenetic trees, only sequences representing the allelic variations of *S. pneumoniae* are shown (Fig. 2 and Fig. S2).

First, sequences covering a 761-bp segment of *tuf* were used for phylogenetic analysis (Fig. S2). All *S. pseudopneumoniae* strains clustered together, except for one strain (2987-98), which clustered with the *tuf*-14 allele of *S. pneumoniae* (represented by strains 578 and CCRI-14740). Within the *S. pseudopneumoniae* group, all *tuf* sequences were 100% identical. However, one *S. pneumoniae* isolate (CCRI-18123) clustered with the *S. pseudopneumoniae* group (bootstrap value of 84%). Furthermore, it was difficult to assign several isolates to a species (see the top of the tree in Fig. S1). To clarify the species identification of these strains, optochin and bile solubility tests were performed, and the test results were compared with those obtained for known *S. pseudopneumoniae*, *S. pneumoniae*, *S. mitis* and *S. oralis* strains (Table 1). All strains were correctly identified, except for strains CCRI-8984, CCRI-14743, and CCRI-18123. In fact, these three strains were bile-insoluble, resistant to optochin when incubated in CO_2, but susceptible to optochin when incubated in air. Thus, optochin and bile solubility phenotypes strongly suggest that strains CCRI-8984, CCRI-14743, and CCRI-18123, initially identified as *S. pneumoniae*, are indeed representatives of *S. pseudopneumoniae*. Consequently, the *tuf*-based tree did not allow clear differentiation of *S. pseudopneumoniae* strains from some atypical *S. pneumoniae* and *S. mitis* strains. Although the usefulness of *tuf* sequences for the identification of 28 streptococcal species was previously described [15], distinguishing between *S. pseudopneumoniae* and *S. pneumoniae* appears to be difficult with the use of *tuf* gene. In fact, the similarity in *tuf* sequences between *S. pseudopneumoniae* and *S. pneumoniae* ranged from 98.6% to 99.9%.

Second, a 756-bp segment of *recA* from the 334 streptococcal isolates was analysed. *S. pneumoniae* and *S. pseudopneumoniae* clustered together (bootstrap value of 88%) (Fig. 2b). All *S. pseudopneumoniae* strains have highly conserved sequences for *recA* gene. Furthermore, *S. pseudopneumoniae* strains CCRI-8984, CCRI-14743 and CCRI-18123, initially identified as *S. pneumoniae* by phenotypic tests routinely used in clinical laboratories, shared 100% *recA* identity with the *S. pseudopneumoniae* type strain. All *S. pseudopneumoniae* strains (including the three strains re-identified in this study) grouped into a monophyletic subcluster with a 99% bootstrap value at the node. Comparison of the *recA* sequences from strains of *S. pseudopneumoniae*, *S. pneumoniae*, *S. mitis*, and *S. oralis* revealed interspecies identities ranging from 89.2% to 97.6%. Moreover, all *S. pseudopneumoniae* isolates analysed in this study had the same three single-nucleotide polymorphisms (including the Thr275 → Ala signature). On the basis of these data, *S. pseudopneumoniae* could be clearly distinguished from *S. pneumoniae* as well as from other mitis group streptococci by *recA* sequence analysis.

The *tuf* and *recA* sequences analysed from the 334 streptococcal isolates included sequences from 143 and 129

S. pneumoniae strains isolated from lower respiratory tract and blood specimens, respectively. Among these *S. pneumoniae* isolates, three of 143 from lower respiratory tract specimens were re-identified as *S. pseudopneumoniae*, and none of 129 from blood specimens was identified as *S. pseudopneumoniae*, suggesting that *S. pseudopneumoniae* has a low invasive capacity. This further supports previous studies showing the presence of *S. pseudopneumoniae* in the lower respiratory tract [2,4,7].

Real-time PCR assay

For development of the *S. pseudopneumoniae* real-time PCR assay, primers were designed to specifically amplify both *S. pseudopneumoniae* and *S. pneumoniae*. One specific locked nucleic acid-Taqman probe was designed to specifically detect *S. pseudopneumoniae* strains. The position of primers and probe on the *recA* gene are shown in Fig. S2. The analytical specificity of the real-time PCR assay was evaluated with 1 ng of purified genomic DNA from 11 *S. pseudopneumoniae* strains, 12 *S. pneumoniae* strains (including nine atypical pneumococci), and four strains each of *S. mitis* and *S. oralis* (corresponding to different clusters on *recA* gene). The analytical specificity of the assay was 100% for *S. pseudopneumoniae*. In order to determine the detection limit of the real-time PCR assay, serial dilutions of purified genomic DNA from four *S. pseudopneumoniae* strains were tested. The assay showed high sensitivity, with a limit of detection equivalent to <10 genomic copies for *S. pseudopneumoniae*. Considering that all *S. pseudopneumoniae* strains studied here were only isolated from North America, more strains from other countries need to be studied to better determine the ubiquity of our real-time PCR. The development of a PCR assay for the specific identification of *S. pseudopneumoniae* could contribute to refinement of the epidemiology of respiratory tract infections. Accurate identification of *S. pseudopneumoniae* will help to establish its prevalence in clinical settings.

Conclusion

This report describes a genetic target (*recA*) for the specific identification of *S. pseudopneumoniae* that allows good discrimination of *S. pseudopneumoniae* from closely related species. Moreover, in the present article, we describe for the first time the development and evaluation of a locked nucleic acid-Taqman probe-based real-time PCR for the detection and identification of *S. pseudopneumoniae*. This real-time PCR assay provides a rapid, specific, and sensitive method for the identification of *S. pseudopneumoniae*, thus rendering it feasible and attractive for diagnostic use or large-scale screening to determine the prevalence of this species in respiratory tract infections.

Conflict of Interest

None of the authors reports any conflict of interest.

Acknowledgements

We are grateful to M.-C. Hélie for skilful technical assistance. These results were presented, in part, at the 106th general meeting of the American Society for Microbiology, Orlando, Florida, USA, 2006.

Transparency Declaration

This study was supported by Genome Canada and Génome Québec.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree of clinical isolates based on *tuf* gene sequences.

Figure S2. Position of primers and probe used for real-time PCR.

Table S1. Characteristics of bacterial strains used in this study.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

1. Euzéby J. Validation of publication of new names and new combinations previously effectively published outside the IJSEM. *Int J Syst Evol Microbiol* 2005; 55: 1–2.
2. Arbique JC, Poyart C, Trieu-Cuot P *et al.* Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. *J Clin Microbiol* 2004; 42: 4686–4696.
3. Díaz E, López R, García JL. Role of the major pneumococcal autolysin in the atypical response of a clinical isolate of *Streptococcus pneumoniae*. *J Bacteriol* 1992; 174: 5508–5515.

4. Carvalho MD, Tondella ML, McCausstland K *et al.* Evaluation and improvement of real-time PCR detection of assays to *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. *J Clin Microbiol* 2007; 45: 2460–2466.
5. Cochetti I, Vecchi M, Mingoia M *et al.* Molecular characterization of pneumococci with efflux-mediated erythromycin resistance and identification of a novel *mef* gene subclass, *mef*(I). *Antimicrob Agents Chemother* 2005; 49: 4999–5006.
6. Harf-Monteil C, Granello C, Le Brun C, Monteil H, Riegel P. Incidence and pathogenic effect of *Streptococcus pseudopneumoniae*. *J Clin Microbiol* 2006; 44: 2240–2241.
7. Keith ER, Podmore RG, Anderson TP, Murdoch DR. Characteristics of *Streptococcus pseudopneumoniae* isolated from purulent sputum samples. *J Clin Microbiol* 2006; 44: 923–927.
8. Keith ER, Murdoch DR. Antimicrobial susceptibility profile of *Streptococcus pseudopneumoniae* isolated from sputum. *Antimicrob Agents Chemother* 2008; 52: 2998.
9. Richter SS, Heilmann KP, Dohrn CL, Riahi F, Beekmann SE, Doern GV. Accuracy of phenotypic methods for identification of *Streptococcus pneumoniae* isolates included in surveillance programs. *J Clin Microbiol* 2008; 46: 2184–2188.
10. Llull D, López R, García E. Characteristic signatures of the *lytA* gene provide a basis for rapid and reliable diagnosis of *Streptococcus pneumoniae* infections. *J Clin Microbiol* 2006; 44: 1250–1256.
11. Volturo GA, Low DE, Aghababian R. Managing acute lower respiratory tract infections in an era of antibacterial resistance. *Am J Emerg Med* 2006; 24: 329–342.
12. Whatmore AM, Efstratiou A, Pickerill AP *et al.* Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: characterization of 'atypical' pneumococci and organisms allied to *S. mitis* harbouring *S. pneumoniae* virulence factor-encoding genes. *Infect Immun* 2000; 68: 1374–1382.
13. Kawamura Y, While RA, Shu SE, Ezaki T, Hardie JM. Genetic approaches to the identification of the mitis group within the genus *Streptococcus*. *Microbiology* 1999; 145: 2605–2613.
14. Paradis S, Boissinot M, Paquette N *et al.* Phylogeny of the *Enterobacteriaceae* based on genes encoding elongation factor Tu and F-ATPase β-subunit. *Int J Syst Evol Microbiol* 2005; 55: 2013–2025.
15. Picard FJ, Ke D, Boudreau DK *et al.* Use of *tuf* sequences for genus-specific PCR detection and phylogenetic analysis of 28 streptococcal species. *J Clin Microbiol* 2004; 42: 3686–3695.
16. Thompson CC, Thompson FL, Vandemeulebroecke K, Hoste B, Dawyndt P, Swings J. Use of *recA* as an alternative phylogenetic marker in the family *Vibrionaceae*. *Int J Syst Evol Microbiol* 2004; 54: 919–924.
17. Clarridge JE III. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev* 2004; 17: 840–862.
18. Woo PC, Lau SK, Teng JL, Tse H, Yuen KY. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin Microbiol Infect* 2008; 14: 908–934.
19. Balsalobre L, Hernández-Madrid A, Llull D *et al*. Molecular characterization of disease-associated streptococci of the mitis group that are optochin susceptible. *J Clin Microbiol* 2006; 44: 4163–4171.
20. Blackwood KS, He C, Gunton J, Turenne CY, Wolfe J, Kabani AM. Evaluation of *recA* sequences for identification of *Mycobacterium* species. *J Clin Microbiol* 2000; 38: 2846–2852.
21. Gutacker M, Conza N, Benagli C *et al.* Population genetics of *Vibrio vulnificus*: identification of two divisions and a distinct eel-pathogenic clone. *Appl Environ Microbiol* 2003; 69: 3203–3212.
22. Stine OC, Sozhamannan S, Gou Q, Zheng S, Morris JG, Johnson JA. Phylogeny of *Vibrio cholerae* based on *recA* sequence. *Infect Immun* 2000; 68: 7180–7185.
23. Torriani S, Felis GE, Dellaglio F. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Appl Environ Microbiol* 2001; 67: 3450–3454.