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Genetic approaches to the identification of the mitis group within the genus *Streptococcus*

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The usefulness and reliability of partial sequence analysis of the manganese-dependent superoxide dismutase gene (*sodA*), autolysin (*lytA*) gene amplification and species-specific PCR based on the D-alanine:D-alanine ligase (*ddl*) gene for differentiating each member of the mitis group of the genus *Streptococcus* was investigated. On the phylogenetic tree based on *sodA* partial sequences (366 bp) from 96 strains, including all species currently within the mitis group isolated in different geographic areas (mainly Japan and the UK), eight well separated clusters were generated corresponding to recognized species, and all strains fell into those clusters to which they had also been assigned by DNA–DNA hybridization. The *Streptococcus pneumoniae* sub-cluster was located within the *Streptococcus mitis* cluster, but the *sodA* gene of *S. pneumoniae* was very conserved and therefore could be separated from all other species examined. Furthermore, the *lytA* gene amplification approach could also be used to differentiate *S. pneumoniae* from other species. The species-specific amplification product of the *ddl* gene was successfully detected in *Streptococcus sanguinis* and *Streptococcus gordonii*, but failed to be detected in some strains of *Streptococcus oralis* including the type strain and *S. mitis*. We conclude that the partial sequence analysis of the *sodA* gene could be applied globally as a reliable and easy method for the accurate identification of all species currently within the mitis group.

Keywords: Superoxide dismutase gene, autolysin gene, D-alanine:D-alanine ligase gene, mitis group, *Streptococcus*

INTRODUCTION

Based on 16S rRNA gene sequence analysis, species of the genus *Streptococcus* were separated into six major clusters or species groups (Bentley *et al*., 1991; Kawamura *et al*., 1995). Within these clusters, the mitis group contained several clinically important species, such as *Streptococcus pneumoniae*, *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus sanguinis*. It is well known that members of this group are difficult to identify correctly, and show 40–60% DNA–DNA similarity values with each other. In addition, some species share greater than 99% 16S rRNA sequence homology (Kawamura *et al*., 1995). Biochemical identification schemes for oral or viridans streptococci have been proposed (Kilian *et al*., 1989; Beighton *et al*., 1991); however, these schemes are insufficient for identifying all the currently recognized species within the mitis group. Similarly, commercially available identification kits give limited identification: in a previous study, we evaluated two kinds of identification kits for streptococci (Rapid ID32 Strep. and STREPTOGRAM) and demonstrated that these kits showed less than 79% accuracy within the mitis group (Kawamura, 1996, 1998). Recently, we used the BIOLOG identification system, which employed 95 different reactions and this seemed to be useful. However, even using this system, some strains of *S. mitis* and *S. oralis* were mis-identified (unpublished data). Identification of members of the mitis group remains difficult.

Recently, the epithets of some species within the mitis group have been revised for grammatical reasons: *Streptococcus sanguis* was corrected to *S. sanguinis*, similarly, *Streptococcus parasanguis* was corrected to *Streptococcus parasanguinis* and *Streptococcus crista*

The DDBJ accession numbers of the superoxide dismutase genes described in this paper are shown in Table 1.

Table 1. Strains used and identification results

ND, Not done, negative indicates that no amplification was observed after agarose gel electrophoresis. ATCC, American Type Culture Collection, Manassas, VA, USA; GTC, Gifu Type Culture Collection, Gifu 500–8705, Japan; JCM, Japanese Collection of Micro-organisms, Saitama 351, Japan; NCTC, National Collection of Type Cultures, London NW9 5HT, UK.

Strain	Description/place of isolation	Identification by			PCR amplification of *lytA* gene	*sodA* partial sequence accession number
		DNA–DNA hybridization	*sodA* sequence clustering	*ddl* gene amplification*		
GTC 495^T (=NCTC 12261^T)	*S. mitis* type strain	–	*S. mitis*	*S. mitis*	Negative	AB021542
NCTC 10712	*S. mitis* reference strain	*S. mitis*	*S. mitis*	Negative	Negative	AB021551
O-4	Tooth surface (Japan)	*S. mitis*	*S. mitis*	Negative	Negative	AB021552
O-17	Tooth surface (Japan)	*S. mitis*	*S. mitis*	*S. mitis*	Negative	AB021553
O-24	Tooth surface (Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021554
O-28	Pharynx (Japan)	*S. mitis*	*S. mitis*	*S. mitis*	Negative	AB021555
O-67	Pharynx (Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021556
O-76	Pharynx (Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021557
O-85	Tooth surface (Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021558
O-93	Tooth surface (Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021559
O-99	Pharynx (Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021560
O-107	Tooth surface (Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021561
O-130	Human (details unknown, Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021562
O-131	Human (details unknown, Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021563
O-133	Human (details unknown, Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021564
O-135	Human (details unknown, Japan)	*S. mitis*	*S. mitis*	ND	Negative	AB021565
HV51	Dental plaque (UK)	*S. mitis*	*S. mitis*	Negative	Negative	AB021566
K208	Abscessed tooth (US)	*S. mitis*	*S. mitis*	ND	Negative	AB021567
A17C1	Dental plaque (UK)	*S. mitis*	*S. mitis*	Negative	Negative	AB021568
OS51	Dental plaque (UK)	*S. mitis*	*S. mitis*	Negative	Negative	AB021569
GTC 276^T (=NCTC 11427^T)	*S. oralis* type strain	–	*S. oralis*	Negative	Negative	AB021543
NCTC 7864	*S. oralis* reference strain	*S. oralis*	*S. oralis*	*S. oralis*	Negative	AB021570
O-5	Pharynx (Japan)	*S. oralis*	*S. oralis*	*S. oralis*	Negative	AB021571
O-7	Pharynx (Japan)	*S. oralis*	*S. oralis*	*S. oralis*	Negative	AB021572
O-8	Tooth surface (Japan)	*S. oralis*	*S. oralis*	Negative	Negative	AB021573
O-9	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021574
O-14	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021575
O-15	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021576
O-21	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021577
O-23	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021578
O-27	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021579
O-29	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021580
O-32	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021581
O-33	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021582
O-37	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021583
O-39	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021584
O-46	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021585
O-52	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021586
O-53	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021587
O-54	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021588
O-60	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021589
O-63	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021590
O-69	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021591
O-71	Pharynx (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021592
O-87	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021593
O-100	Tooth surface (Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021594
O-127	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021595
O-150	Human (details unknown, Japan)	*S. oralis*	*S. oralis*	ND	Negative	AB021596
A15C1	Dental plaque (UK)	*S. oralis*	*S. oralis*	ND	Negative	AB021597
B1493	Dental plaque (UK)	*S. oralis*	*S. oralis*	*S. oralis*	Negative	AB021598
B14C3	Dental plaque (UK)	*S. oralis*	*S. oralis*	ND	Negative	AB021599
B88	Dental plaque (UK)	*S. oralis*	*S. oralis*	ND	Negative	AB021600
GPD1	Dental plaque (UK)	*S. oralis*	*S. oralis*	*S. oralis*	Negative	AB021601
OPA1	Dental plaque (UK)	*S. oralis*	*S. oralis*	ND	Negative	AB021602
PC1467	Dental plaque (UK)	*S. oralis*	*S. oralis*	*S. oralis*	Negative	AB021603
PP53	Dental plaque (UK)	*S. oralis*	*S. oralis*	ND	Negative	AB021604
GTC 261^T (=NCTC 7465^T)	*S. pneumoniae* type strain	–	*S. pneumoniae*	Negative	Positive	AB021544
YK-5	Pharynx (Japan)	ND	*S. pneumoniae*	ND	Positive	AB021605
YK-11	Pharynx (Japan)	ND	*S. pneumoniae*	ND	Positive	AB021606
YK-12	Sputum (Japan)	ND	*S. pneumoniae*	ND	Positive	AB021607
YK-14	Pharynx (Japan)	ND	*S. pneumoniae*	ND	Positive	AB021608
YK-20	Trachea (Japan)	ND	*S. pneumoniae*	ND	Positive	AB021609
661 (serotype 9)	Cerebrospinal fluid (UK)	ND	*S. pneumoniae*	ND	Positive	AB021610
653 (serotype 14)	Blood (UK)	ND	*S. pneumoniae*	ND	Positive	AB021611
1510 (serotype 9N)	Cerebrospinal fluid (UK)	ND	*S. pneumoniae*	ND	Positive	AB021612
1293 (serotype 6B)	Blood (UK)	ND	*S. pneumoniae*	ND	Positive	AB021613

Table 1 (*cont.*)

Strain	Description/place of isolation	Identification by			PCR amplification of *lytA* gene	*sodA* partial sequence accession number
		DNA–DNA hybridization	*sodA* sequence clustering	*ddl* gene amplification*		
1454 (serotype 9)	Blood (UK)	ND	*S. pneumoniae*	ND	Positive	AB021614
1565 (serotype 14)	Cerebrospinal fluid (UK)	ND	*S. pneumoniae*	ND	Positive	AB021615
872 (serotype 14)	Blood (UK)	ND	*S. pneumoniae*	ND	Positive	AB021616
1639 (serotype 3)	Blood (UK)	ND	*S. pneumoniae*	ND	Positive	AB021617
3051 (serotype 19F)	Human (details unknown, UK)	ND	*S. pneumoniae*	ND	Positive	AB021618
3203 (serotype 19F)	Human (details unknown, UK)	ND	*S. pneumoniae*	ND	Positive	AB021619
GTC 848^T (=JCM 10158^T)	*S. peroris* type strain	–	*S. peroris*	Negative	Negative	AB021545
O-91	Pharynx (Japan)	*S. peroris*	*S. peroris*	ND	ND	AB021620
O-105	Tooth surface (Japan)	*S. peroris*	*S. peroris*	ND	Negative	AB021621
GTC 849^T (=JCM 10157^T)	*S. infantis* type strain	–	*S. infantis*	Negative	Negative	AB021546
O-103	Pharynx (Japan)	*S. infantis*	*S. infantis*	ND	ND	AB021622
O-134	Pharynx (Japan)	*S. infantis*	*S. infantis*	ND	ND	AB021623
GTC 497^T (=ATCC 10558^T)	*S. gordonii* type strain	–	*S. gordonii*	*S. gordonii*	Negative	AB021547
O-138	Human (details unknown, USA)	*S. gordonii*	*S. gordonii*	*S. gordonii*	ND	AB021624
O-149A	Unknown (Japan)	*S. gordonii*	*S. gordonii*	*S. gordonii*	ND	AB021625
GTC 631^T (=NCTC 12497^T)	*S. cristatus* type strain	–	*S. cristatus*	Negative	Negative	AB021548
CC5A	*S. cristatus* reference strain	ND	*S. cristatus*	ND	ND	AB021626
AK1	*S. cristatus* reference strain	ND	*S. cristatus*	ND	ND	AB021627
CR3	*S. cristatus* reference strain	ND	*S. cristatus*	ND	ND	AB021628
GTC 217^T (=ATCC 10556^T)	*S. sanguinis* type strain	–	*S. sanguinis*	S. sanguinis	Negative	AB021549
O-19	Tooth surface (Japan)	*S. sanguinis*	*S. sanguinis*	*S. sanguinis*	ND	AB021629
O-25	Tooth surface (Japan)	*S. sanguinis*	*S. sanguinis*	*S. sanguinis*	ND	AB021630
O-59	Human (details unknown, Japan)	*S. sanguinis*	*S. sanguinis*	*S. sanguinis*	ND	AB021631
O-148A	Unknown (Japan)	*S. sanguinis*	*S. sanguinis*	*S. sanguinis*	ND	AB021632
GTC 498^T (=ATCC 15912^T)	*S. parasanguinis* type strain	–	*S. parasanguinis*	Negative	Negative	AB021550
O-10	Pharynx (Japan)	*S. parasanguinis*	*S. parasanguinis*	ND	ND	AB021633
O-31	Human (details unknown, Japan)	*S. parasanguinis*	*S. parasanguinis*	ND	ND	AB021634
O-75	Pharynx (Japan)	*S. parasanguinis*	*S. parasanguinis*	ND	ND	AB021635
O-145A	Unknown (Japan)	*S. parasanguinis*	*S. parasanguinis*	ND	ND	AB021636
O-151A	Unknown (Japan)	*S. parasanguinis*	*S. parasanguinis*	ND	ND	AB021637

* Dependent on the species identified by DNA–DNA hybridization, we selected one of the species-specific primer sets specific for *S. mitis*, *S. oralis*, *S. gordonii* or *S. sanguinis*.

was corrected to *Streptococcus cristatus* (Truper & De Clari, 1997). We have used the revised names throughout this report. In the 1990s, four new species were described within the mitis group, *S. cristatus*, *S. parasanguinis*, *S. peroris* and *S. infantis*, giving a total of nine species classified within this group (Handley *et al*., 1991; Whiley *et al*., 1990; Kawamura *et al*., 1998). Due to the addition of these new species, we were strongly aware of the potential confusion surrounding the identification of members of the mitis group and were therefore motivated to establish a reliable, globally acceptable and easy practical identification method.

To this end, DNA probes were designed from 16S rRNA sequences. Even though some members of the mitis group share greater than 99% sequence similarities in their 16S rRNA gene, it was possible to design several DNA probes which could differentiate between the type strains of *S. mitis*, *S. oralis*, *Streptococcus gordonii*, *S. cristatus*, *S. sanguinis* and *S. parasanguinis*. When we applied these DNA probes to our clinical strains that had been identified, in advance, by DNA–DNA hybridization, all strains of *S. gordonii*, *S. cristatus*, *S. sanguinis* and *S. parasanguinis* could be correctly identified, although significantly, some clinical strains of *S. oralis* and *S. mitis* were mis-identified. From these results, we concluded that our 16S rRNA gene probe could not be applied unambiguously to the identification of *S. mitis* and *S. oralis* (unpublished data).

Identification on the basis of whole-cell protein profile comparison has also been investigated as a taxonomic tool. In a previous study, we found some clinical strains not included within the same cluster as their respective type strain (unpublished data). Similar data were published by Vandamme *et al*. (1998). According to their report, they observed that *S. mitis* biovar 2 strains were neither in the same cluster as *S. mitis* biovar 1 strains nor formed an independent cluster, and in fact migrated into several other species clusters, that included *S. oralis*, *S. cristatus* and *S. parasanguinis*. Furthermore, while the majority of strains of *S. sanguinis* formed a single cluster, this excluded the type strain of *S. sanguinis*, which was relatively close to the *S. mitis* biovar 1 cluster. From these results, it was apparent that applying protein profiles to the identification of the members of the mitis group would prove problematic.

Recently, some genetic methods for the identification of members of the mitis group at the species level have been proposed by other researchers, such as species-specific PCR primers based on the *ddl* gene and comparative analysis of the partial sequences of the manganese-dependent superoxide dismutase (*sodA*) gene (Garnier *et al*., 1997; Poyart *et al*., 1998). In this study, we have investigated the reliability and usefulness of these genetic methods by using 96 human clinical strains isolated from different geographic areas. Finally, we confirm that the *sodA* partial sequence analysis method is a reliable

and useful method for accurate identification of members of the mitis group.

METHODS

Bacterial strains. The type strains used in this study are *S. mitis* (GTC 495^T = NCTC 12261^T), *S. oralis* (GTC 276^T = NCTC 11427^T), *S. pneumoniae* (GTC 261^T = NCTC 7465^T), *S. gordonii* (GTC 497^T = ATCC 10558^T), *S. cristatus* (GTC 631^T = NCTC 12497^T), *S. sanguinis* (GTC 217^T = ATCC 10556^T), *S. parasanguinis* (GTC 498^T = ATCC 15912^T), *S. peroris* (GTC 848^T = JCM 10158^T) and *S. infantis* (GTC 849^T = JCM 10157^T). We also used some reference strains and 82 human clinical strains mainly isolated in Japan and the UK (Table 1). All strains were grown on Todd–Hewitt broth (Difco) under aerobic conditions at 37 °C after confirming purity on Columbia blood (5 % defibrinated sheep blood) agar plates (bioMérieux).

DNA–DNA hybridization. DNA from all strains was prepared by a standard procedure (Marmur, 1961) with minor modification (Ezaki *et al*., 1983). DNA from each type strain was labelled with photobiotin (Sigma) and microplate quantitative DNA–DNA hybridization was carried out according to previously described methods (Ezaki *et al*., 1988, 1989). Briefly, purified DNA (100 μg ml^{-1}) of each strain was heat denatured and then diluted to 10 μg ml^{-1} with ice cold PBS (pH 7·4) containing 0·1 M $MgCl_2$. The diluted DNA solution was distributed into a microplate (Maxsorp; Inter Med) at 100 μl per well, and the plate incubated at 30 °C for 12 h. The solution was discarded and the plate dried at 60 °C. The plate was prehybridized for 30 min and then hybridized in the presence of 2 × SSC and 50 % formamide, with biotin-labelled DNA at 31 °C for 2 h. The plate was washed three times with 1 × SSC and 100 μl streptavidin–β-D-galactosidase (diluted 1:1000 with 0·5 % BSA in PBS; Gibco-BRL) was added to each well. The plate was incubated at 37 °C for 30 min and washed three times with 1 × SSC. Then 100 μl of the substrate (100 μg 4-methylumbelliferyl-β-D-galactopyranoside ml^{-1}; Sigma) was added to each well and the fluorescence intensity was measured by Cytofluor (model 2350; Millipore). The species name of an isolate was determined if the DNA strongly hybridized and showed greater than 70 % similarity value with the DNA of only one type strain.

PCR amplification of part of the *sodA* gene. An internal portion of the *sodA* gene was amplified by PCR using our designed primer set (SOD-UP; 5′-biotin-TRCAYCATGAY-AARCACCAT-3′ and SOD-DOWN; 5′-ARRTARTAMGC-RTGYTCCCARACRTC-3′). Each PCR reaction (100 μl) contained 1 × PCR buffer (Pharmacia Biotech), 100 μM dNTPs, 0·1 μM and 0·2 μM biotin-labelled (SOD-UP) primer and non-labelled (SOD-DOWN) primer, respectively, 20 ng template DNA and 1 U *Taq* polymerase (Pharmacia Biotech). PCR was carried out on a thermal cycler (model 2400, Perkin Elmer) as follows: 35 cycles of 30 sec at 94 °C, 1 min at 50 °C and 1 min at 72 °C, and a final cycle of 1 min at 50 °C followed by 10 min at 72 °C.

Determination and analysis of *sodA* partial sequences. After confirming the presence of a single 435 bp amplification product of the *sodA* gene on 1 % agarose gels, the sequence was determined using a Pharmacia automatic sequencer (ALF express) with an auto load sequencing kit (Pharmacia Biotech). Briefly, the avidin-conjugate comb was soaked in the PCRed solution to capture the biotin-labelled PCR product. Then the comb was washed in 0·1 M NaOH solution to denature the amplicon and to remove other substances of the PCR reaction mixture. The sequencing reaction was carried out on the comb using T7 polymerase and Cy5-labelled primer (the sequence was the same as for the SOD-DOWN primer). The reacted comb was applied directly onto the sequencing gel and the sequencer run for 10 h.

Phylogenetic analysis of *sodA* partial sequences. CLUSTAL W software originally described by Thompson *et al*. (1994) was used to align the sequences, and the phylogenetic distances were calculated using the neighbour-joining method. The phylogenetic tree was drawn using TreeView software (Page, 1996). The *sodA* partial sequence of *Streptococcus agalactiae* was obtained from the DNA Database of Japan (DDBJ, accession number is Z95893) and used as the outgroup.

D-Alanine:D-alanine ligase gene specific PCR. Species-specific PCR for *S. mitis*, *S. oralis*, *S. gordonii* and *S. sanguinis* based on the D-alanine:D-alanine ligase gene (*ddl* gene) was carried out according to Garnier *et al*. (1997). Ten microlitres of each PCR reaction was applied to a 1 % agarose gel and electrophoresed.

Autolysin gene (*lytA*) specific PCR. *lytA*-specific PCR for the identification of *S. pneumoniae* was carried out according to Gillespie *et al*. (1994). PCR amplification was confirmed by electrophoresis through 1 % agarose gels.

RESULTS

DNA–DNA hybridization

To determine species identification, we applied our DNA–DNA hybridization method to all strains of unidentified isolates (a total of 69 isolates). All identification results are shown in Table 1.

ddl gene amplification

We confirmed the specificity of each species-specific primer set based on the *ddl* gene by using the type strains of members of the mitis group, except *S. oralis* (Table 1). We also applied this method to some of the *S. mitis*, *S. oralis*, *S. gordonii* and *S. sanguinis* strains. All strains of *S. gordonii* (three strains including the type strain) and *S. sanguinis* (five strains including the type strain) were positive. However, the *S. oralis* type strain and one of our clinical strains were negative. The type strain of *S.*

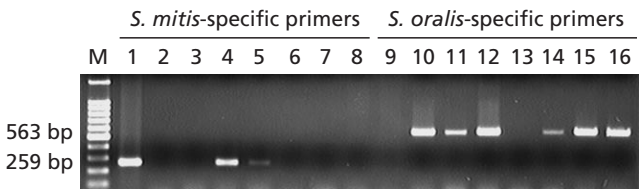

Fig. 1. Results of *ddl* gene amplification using *S. mitis*- or *S. oralis*-specific primers. The molecular size marker is the 100 bp ladder (lane M). Lanes: 1, *S. mitis* GTC 495^T; 2, *S. mitis* NCTC 10712; 3, *S. mitis* O-4; 4, *S. mitis* O-17; 5, *S. mitis* O-28; 6, *S. mitis* OS51; 7, *S. mitis* HV51; 8, *S. mitis* A17C1; 9, *S. oralis* GTC 276^T; 10, *S. oralis* NCTC 7864; 11, *S. oralis* O-5; 12, *S. oralis* O-7; 13, *S. oralis* O-8; 14, *S. oralis* PC1467; 15, *S. oralis* B1493; 16, *S. oralis* GPD1.

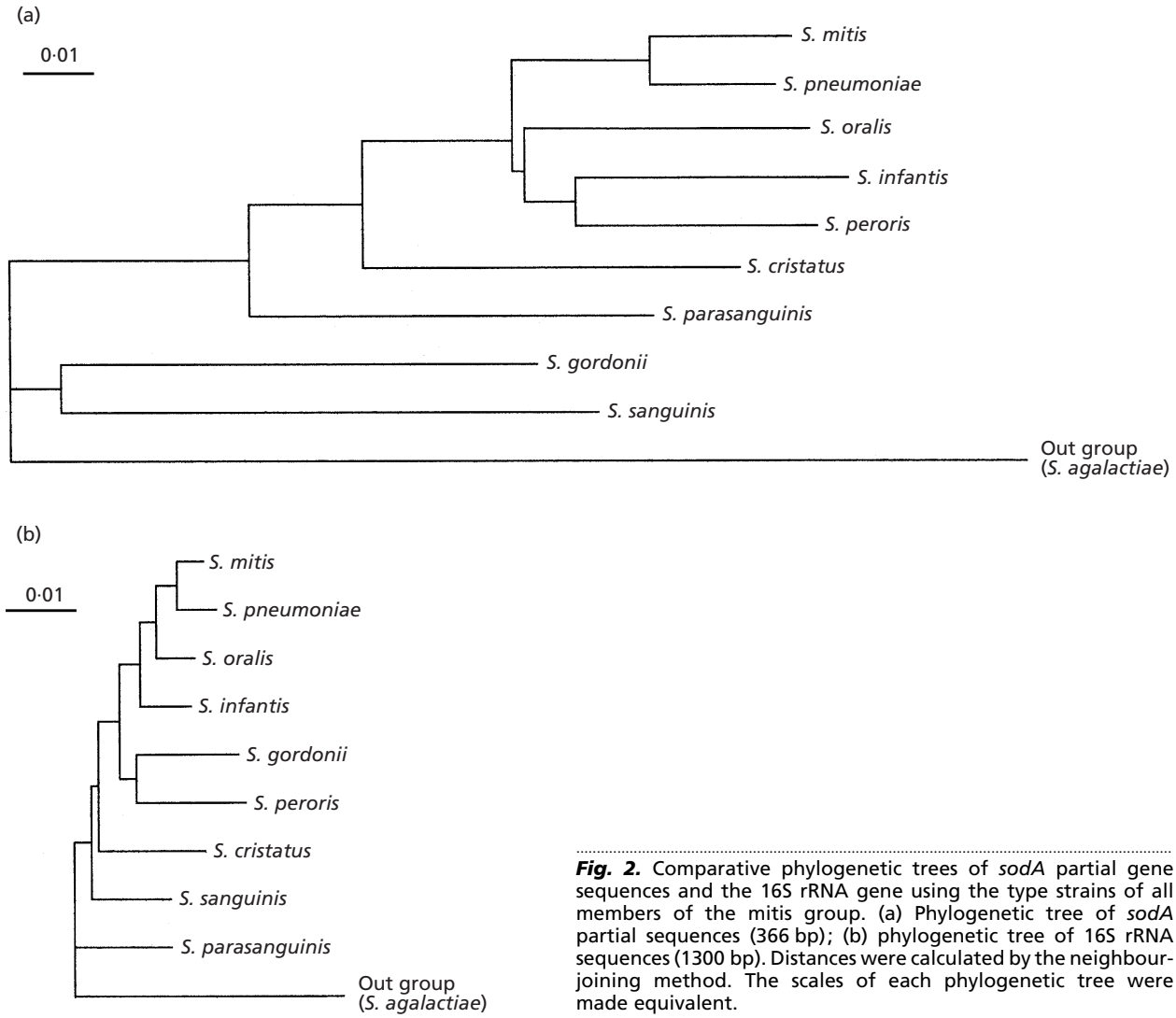

Fig. 2. Comparative phylogenetic trees of *sodA* partial gene sequences and the 16S rRNA gene using the type strains of all members of the mitis group. (a) Phylogenetic tree of *sodA* partial sequences (366 bp); (b) phylogenetic tree of 16S rRNA sequences (1300 bp). Distances were calculated by the neighbour-joining method. The scales of each phylogenetic tree were made equivalent.

mitis gave a positive result. However, one reference strain (NCTC 10712) and four of six clinical strains tested were negative (Fig. 1, Table 1).

lytA gene amplification

lytA gene amplification gave amplicons from all *S. pneumoniae* strains including the type strain, while all other strains, including those of closely related species *S. mitis* and *S. oralis*, gave completely negative results (data not shown, the results are summarized in Table 1)

Phylogenetic analysis of *sodA* partial sequences

The phylogenetic tree of partial sequences of *sodA* was compared with that of 16S rRNA sequences for the type strains. It can be seen that the evolutionary rate of the *sodA* partial gene is much faster than that of the 16S rRNA gene (Fig. 2). We took this to indicate that partial *sodA* sequencing would be useful for differentiating genetically closely related organisms, and decided to apply *sodA* partial sequencing to the main collection of isolates. We determined the partial sequences of the *sodA* gene from 96 strains including all species currently within the mitis group and constructed a phylogenetic tree using the neighbour-joining method. Eight clusters were clearly generated corresponding to recognized species as confirmed by DNA–DNA hybridization (Fig. 3). With respect to the Japanese and UK isolates of *S. mitis* and *S. oralis*, each species formed its own cluster with no evidence of geographic variation. *S. pneumoniae* strains did not form an independent cluster but formed a sub-cluster within the *S. mitis* cluster on the *sodA* phylogenetic tree.

S. pneumoniae-specific bases within *sodA*

S. pneumoniae strains did not cluster independently on the *sodA* phylogenetic tree. However, *sodA* partial sequences of all *S. pneumoniae* strains were almost identical and some species-specific base differences were detected that were helpful for differentiating

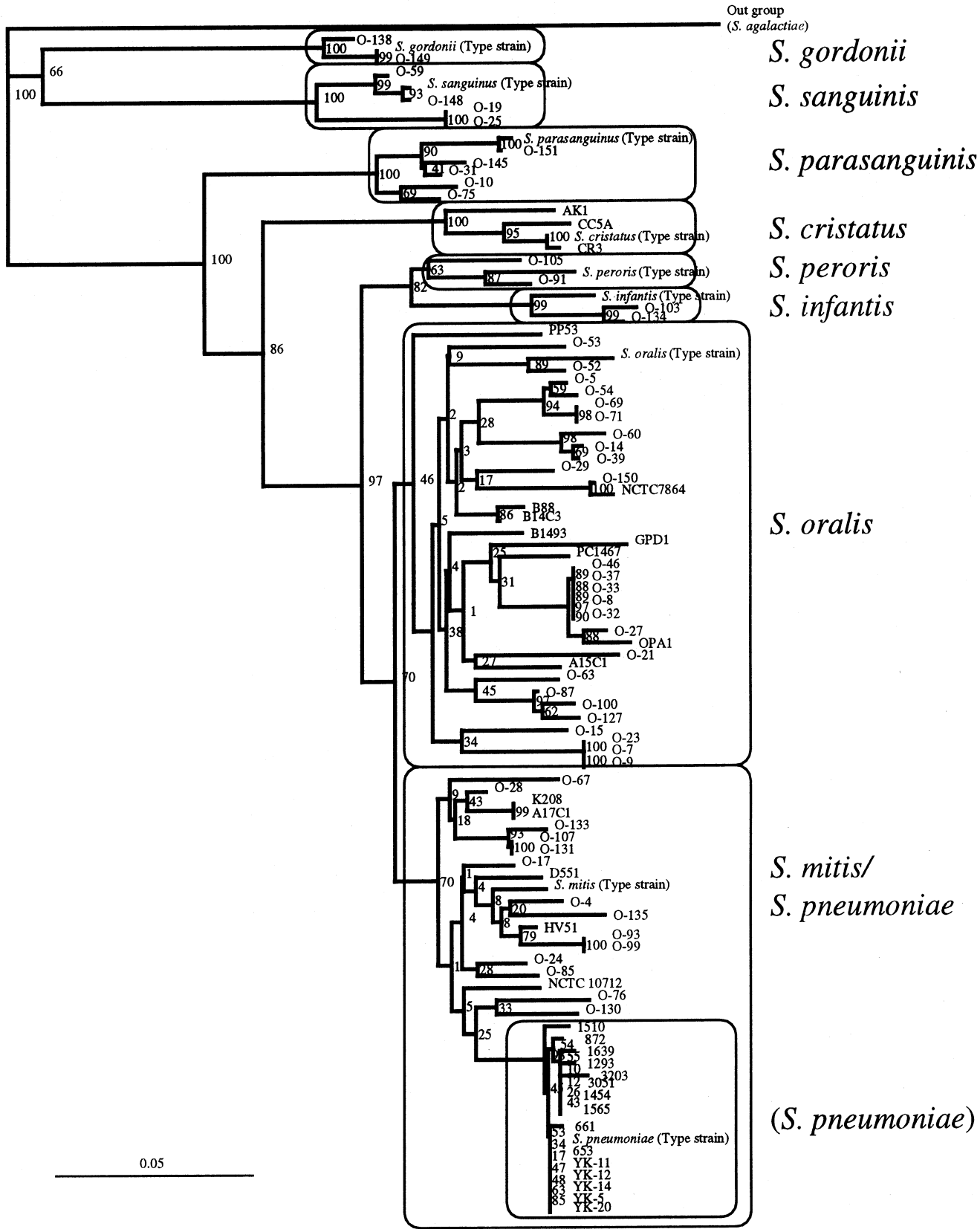

Fig. 3. Phylogenetic tree based on *sodA* partial sequences (366 bp). Distances were calculated by the neighbour-joining method. The numbers at the nodes are bootstrap values (based on 100 samplings). Each member species of the mitis group formed a separate cluster, except *S. pneumoniae*. All *S. pneumoniae* strains were located within the *S. mitis* cluster as a distinct sub-cluster.

Table 2. Some bases of the *sodA* gene helpful in differentiating *S. pneumoniae* from other species

Numbers in parentheses show the numbers of strains with each base.

Species	Alignment position				
	31	**33**	**132**	**174**	**288**
S. pneumoniae	T	A	C	C	T
S. mitis	C	T/A (18/2)	T/C (16/4)	T	A/C (12/8)
S. oralis	C	T	T/C (35/1)	T/G/A (30/4/2)	A/C (28/8)
S. peroris	C	T	T	T	A
S. infantis	C	T	T	T	A
S. gordonii	C	T	T	A	C
S. cristatus	C	T	T	T	T
S. sanguinis	C	T	T	A	C
S. parasanguinis	C	T	T	A	C

S. pneumoniae from other species including *S. mitis* (Table 2).

DISCUSSION

In 1997, Garnier and co-workers developed a promising method for the identification of *S. mitis*, *S. oralis*, *S. gordonii* and *S. sanguinis* (Garnier *et al.*, 1997). According to their report, they used species-specific PCR primers based on the *ddl* gene, and could exploit size polymorphisms between the species tested. If this method worked well, it was expected to be useful. However, no amplicon was observed here when we applied *S. oralis*-specific primers (the G primer pair of Garnier *et al.*) to the type strain of *S. oralis*. We could not compare this result with their report, because the previous authors did not use the type strain of *S. oralis* in their study. We had applied milder PCR conditions [5 °C lower annealing temperature (51 °C) than that of Garnier *et al.*], although even under those conditions we did not get a positive result from the type strain of *S. oralis*. Furthermore, we used two different laboratory versions of the type strain of *S. oralis* (Gifu laboratory strain GTC 276^T, which was directly received from NCTC as NCTC 11427^T, and another from London, strain LVG1^T). Neither strain yielded a positive result. From these observations, we conclude that the reported *S. oralis*-specific primers based on the *ddl* gene could not amplify DNA of the *S. oralis* type strain. Similarly, one reference strain of *S. mitis* (NCTC 10712) did not give a positive result when using *S. mitis*-specific primers (the F primer pair of Garnier *et al.*). Finally, we applied these PCR primers to eight strains of each of *S. oralis* and *S. mitis*, and could not obtain amplification from two strains of *S. oralis* (the type strain and one clinical strain) and five strains of *S. mitis* (NCTC 10712 and four clinical strains). We therefore abandoned the idea of using these primers for species identification.

After developing *lytA*-specific PCR (Rudolph *et al.*, 1993), many researchers have successfully detected or identified *S. pneumoniae* by this method using purified DNA as a template (Gillespie *et al.*, 1994; Hassan-King *et al.*, 1994; Ubukata *et al.*, 1996). Recently, Whatmore *et al.* (1996) reported that a subset of *S. mitis* and *S. oralis* strains isolated were positive for this gene by PCR. However we have applied this method to more than 20 strains of *S. pneumoniae* and more than 70 strains of *S. mitis* or *S. oralis*, and did not observe any positive results from *S. mitis* nor *S. oralis* strains. From these results, we thought that the *lytA*-specific PCR might be helpful, in many cases, for identification of *S. pneumoniae*.

Recently, several identification methods using gene sequence data have been established, such as DNA gyrase B subunit (Yamamoto & Hirayama, 1995, 1996), heat-shock protein 60 (Goh *et al.*, 1996, 1998) and the *sodA* gene (Smith & Doolittle, 1992; Zolg & Philippi-Schulz, 1994; Poyart *et al.*, 1995, 1998). Of these, the *sodA* partial gene sequencing seemed to be relatively useful, because less than 400 bp is enough to determine the phylogenetic position of a strain. Therefore, we selected this gene for the identification of members of the mitis group.

When the *sodA* phylogenetic tree from each species type strain within the mitis group was constructed, it became apparent that the evolutionary rate of the *sodA* gene was much faster than that of 16S rRNA sequence (Fig. 2). Thus, we suspected that this gene would be useful in differentiating genetically closely related organisms. However, it was possible that there might be too much variation within the same species, possibly due to geographic differences, for this approach to be practicable. To test the method, clinical strains from different areas, mainly from Japan and the UK, were examined but failed to reveal any obvious geographic variation (Fig. 3). For these reasons, we believe that this method could be applied globally.

Some bootstrap values of the branching point of each species on the *sodA* phylogenetic tree were not very high (the lowest is 66%, at the *S. gordonii* and *S. sanguinis*

branching point, Fig. 3). However, before making the final phylogenetic tree (Fig. 3), we remade the tree each time we collected new sequence data, and each time the species separated in the same way as Fig. 3. From this experience, we consider that the branching of each species on the *sodA* tree is reproducible.

All strains of each member species of the mitis group formed a single cluster. However, many strains of the same species showed several base substitutions (maximum 31 bases) within the 366 bp region of the *sodA* gene examined, while the gene of *S. pneumoniae*, by comparison, was very conserved. Eleven of 16 *S. pneumoniae* strains showed completely identical sequences, and only two strains (strains 1510 and 3203) and three strains (strains 1639, 1293 and 661) showed two and one base substitutions, respectively. Similar data have been published by Poyart *et al.* (1998). These authors used eight isolates of *S. pneumoniae* of which only two showed a single base substitution. We cannot explain why the *sodA* gene of *S. pneumoniae* is so conserved, but are encouraged that this conservation is helpful for the identification of *S. pneumoniae* (Table 2).

In this study, we determined the sequences direct from PCR amplicons using a Pharmacia sequencer with an auto-load sequencing kit. By using this method, we could apply the sample onto the sequencer within a day from the bacterial culture without doing any troublesome manipulation, such as ethanol precipitation and prolonged centrifugation of the sample, and could obtain the sequence data the next morning. There are many internet home pages providing a service to enable calculation of phylogenetic relationships, e.g. CLUSTAL W could be carried out on the home page of the DNA Databank of Japan (http://www.ddbj.nig.ac.jp). By using these easy practical methods, we believe that this method could be carried out rapidly in many laboratories.

In view of the data presented above, we believe that the *sodA* partial sequence analysis method could be applied globally as a reliable and practical method for the accurate identification of all species currently within the mitis group.

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