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Identification of Clinically Relevant Viridans Group Streptococci to the Species Level by PCR

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A PCR assay that allows identification of clinically relevant viridans group streptococci (*Streptococcus gordonii*, *S. mitis*, *S. mutans*, *S. oralis*, *S. salivarius*, and *S. sanguis*) to the species level and identification of milleri group streptococci (*S. anginosus*, *S. constellatus*, and *S. intermedius*) to the group level was developed. This assay was based on specific amplification of internal fragments of genes encoding D-alanine:D-alanine ligases which are species specific and ubiquitous in prokaryotes possessing peptidoglycan. The specificity of this assay was tested on 9 reference strains and 91 characterized clinical isolates. This assay offers a specific and rapid alternative to phenotypic or DNA-DNA hybridization methods for identification of clinically relevant viridans group streptococci.

Viridans group streptococci form an important part of the normal flora of the human oral cavity. They are responsible for several infections including purulent infections (14), endocarditis (7), septicemia (4), and meningitis (3). Viridans streptococci do not possess a specific group antigen and show variable reactions with Lancefield antisera (11). Their identification is based on different physiological and biochemical characteristics, but conventional phenotypic identification methods are sometimes unable to differentiate established species. First, not all strains in a species may be positive for a common trait (2, 19), and second, the same strain may give different results with repeated tests in the absence of changes in the corresponding genes (15, 25). Thus, rapid systems using standard phenotypic tests, such as API-20STREP or rapid ID 32 Strep, that are used in clinical laboratories are not totally satisfactory for accurate identification at the species level (16, 18). Species identification of viridans streptococci is useful in cases of infective endocarditis when the patient has relapsed and in cases of positive blood cultures and in assessing the involvement of a given strain in an infection.

PCR has been extensively applied to species identification of infectious agents (8, 13, 21). PCR allows amplification of a preselected DNA region and is a highly specific and sensitive technique (20). In many instances, the target genes are involved in pathogenicity (13). In other cases, the target is a random cloned fragment from a genomic library selected by differential hybridization to the pathogen and its close relatives (23).

In this study, we have selected the gene encoding a D-alanine:D-alanine (D-Ala:D-Ala) ligase which is species specific and ubiquitous in prokaryotes possessing peptidoglycan. The D-Ala:D-Ala ligase catalyzes synthesis of the terminal dipeptide D-alanine–D-alanine of peptidoglycan precursors (26). We have developed a PCR assay which allows identification of six species (*Streptococcus gordonii*, *S. mitis*, *S. mutans*, *S. oralis*, *S. salivarius*, and *S. sanguis*) to the species level and identification of three species (*S. anginosus*, *S. constellatus*, and *S. intermedius*) to the group level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The reference strains of *Streptococcus* spp. used in this study were as follows: *S. anginosus* ATCC 33397, *S. bovis* NCTC 8177, *S. constellatus* ATCC 27823, *S. gordonii* ATCC 10558, *S.*

TABLE 1. Oligodeoxynucleotide primers for the first PCR

Streptococcal species	Size of PCR product (bp)	Oligodeoxynucleotide		Positions[a]	GC content (%)
		Pair	Sequence		
S. mitis	372	A	5′-GTCGAAGGTGATGATATGAC-3′	133–152	50
S. oralis			3′-GACAGTACGCAGTCTTACGTC-5′	488–508	52
S. mutans	351	B	5′-ATTGAAGGCGAGCCTTTAGAAAG-3′	133–155	43
			3′-CTAGGACAATAGCAAC-5′	472–487	43
S. salivarius	331	C	5′-GCAGCAGTAGCAGAGACGCT-3′	154–173	60
			3′-CACGGACGTCTTCAGTACTG-5′	469–488	55
S. gordonii	208	D	5′-GTCGATGGCGAGGATCTAGAGC-3′	133–154	59
S. sanguis			3′-TGCCGAGCGCTCTAACTCCA-5′	325–344	60

[a] Positions were derived from the alignment in Fig. 1.

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                1                                                   50
S. gordonii     TTCCTAGAAG TCCTCAAAAT GCCTTATGTC GGCTGTAATA TTCTATCTTC
S. sanguis      TTCCTAGAAG TGCTCAAAAT GCCTTATGTC GGCTGTAATA TTTTATCTTC
S. mitis        TTCCTTGAAG TTTTGAAAAT GCCTTATGTT GGTTGCAACA TTTTGTCATC
S. oralis       TTTCTTGAAG TTTTGAAAAT GCCTTATGTC GGCTGCAATA TCCTGTCATC
S. anginosus    TTTCTTGAAG TGTTGAAAAT GCCTTATGTG GGTTGCAATA TCCTTTCTTC
S. constellatus TTTCTTGAAG TGTTGAAAAT GCCTTATGTG GGTTGTAATA TCCTTTCTTC
S. intermedius  TTTCTTGAAG TGTTGAAAAT GCCTTATGTT GGCTGCAATA TTCTTTCGTC
S. salivarius   TTCCTCGAAA CCCTCAAATT GCCATATGTG GGTACTAATG TTCTTTCTTC
S. mutans       TTTCTTGAAG TTTTAAAAAT GCCTTATGTG GGAACCAATA TTCTGTCATC
                ** ** ***     * *** * *** *****  **    **   *  * ** **

                51                                                 100
S. gordonii     TAGCCTGGCC ATGGATAAAA TTACGACCAA GCGAGTTCTG GAATCAG...
S. sanguis      TAGCTTAGCC ATGGATAAAA TCACGACCAA GCGAGTGCTG GAATCAG...
S. mitis        TAGCCTTGCC ATGGACAAAA TCACGACCAA GCGTGTGTTA GAATCTG...
S. oralis       AAGTCTTGCC ATGGATAAAA TTACGACCAA GCGTGTTTTA GAATCTG...
S. anginosus    TAGTGTTGCT ATGGACAAAA TCACGACAAA ACGAGTGTTA GCTTCTG...
S. constellatus TAGTGTTGCC ATGGACAAAA TCACAACAAA ACGAGTGTTA GCTTCTG...
S. intermedius  GAGTATTGCG ATGGATAAAA TCACAACTAA ACGTGTGTTA GCTTCTG...
S. salivarius   AAGTGTGGCT ATGGACAAGA TTATGACCAA GCATATTCTT GAAGTTG...
S. mutans       TAGTGTAGCT ATGGATAAGA TTACAACAAA GCAAGTTTTA GAAAGTGCGA
                 **  * **  ***** ** * * *  ** **  *   *  *  *     *

                101                                                150
S. gordonii     CAGGTATTGC TCAAGTACCT TATGTAGCGG TGGTTGATGG TGAGGATCTA
S. sanguis      CAGGAATAGC CCAAGTGCCT TATGTGGCGC TGGTCGATGG CGAGGATCTA
S. mitis        TCGGGATTGC CCAAGTTCCT TATGTGGCCA TTGTCGAAGG TGATGATATG
S. oralis       TAGGGATTGC CCAAGTACCT TATGTGGCTA TCGTCGAAGG TGATGATGTG
S. anginosus    CTGGCATTCC GCAAGTTCCC TATGTAGCAG TGATTGAAGG GGAAAATATA
S. constellatus CTGGCATTCC GCAAGTTCCC TATGTAGCAG TGATTGAAGG GGAAAATATA
S. intermedius  CTGGTATTCC TCAAGTACCG TATGTAGCCG TAGTGGAAGG GGAAGATGTA
S. salivarius   CTGGTGTGCC TCAGGTTGCC TATACAGTCT TCATCGAGGG TGAAGATTTG
S. mutans       CTACTATTCC TCAAGTAGCT TATGTTGCTC TTATTGAAGG CGAGCCTTTA
                      *  *  ** **  *  ***   *    *  * ** **  **   * *

                151                                                200
S. gordonii     GAGCAAAAAA TCCAGGAAAT TGAAGAGAAA CTGTCCTATC CCGTCTTCAC
S. sanguis      GAGCAAAAAA TCCAAGAAAT CGAGGAGAAA TTGACCTATC CAGTCTTCAC
S. mitis        ACTGCTAAAA TTGCTGAAGT TGAAGAAAAA TTGACTTATC CAGTTTTCAC
S. oralis       ACTGCTAAAA TAGCTGAAGT TGAAGAAAAA TTGACTTATC CAGTCTTCAC
S. anginosus    GACGAAAAGA TTGCAGCAGT AGAAGCCAAT CTGACTTATC CAGTTTTTAC
S. constellatus GACGAAAAGA TTGCAGAAGT AGAGGCAAAT CTAACTTATC CAGTTTTTAC
S. intermedius  GAAGAAAAGA TTGCAGAAGT AGAAGTTAAT CTGACTTATC CAGTCTTTAC
S. salivarius   GAAGCAGCAG TAGCAGAGAC GCTTGAAAAA TTGACCTTCC CAGTGTTTGT
S. mutans       GAAAGCAAGT TGGCAGAAGT TGAAGAGAAA TTGATTTATC CTGTATTTGT
                           *    *         *  **   * *  *  * * ** **

                201                                                250
S. gordonii     CAAACCTTCT AATATGGGCT CCAGTGTCGG CATTTCAAAA TCTGATAATC
S. sanguis      CAAACCTTCT AATATGGGCT CCAGTGTCGG CATTTCTAAA TCGGA.GACC
S. mitis        AAAACCATCT AACATGGGTT CTAGTGTCGG TATTTCTAAG TCTGAAAATC
S. oralis       GAAGCCGTCA AACATGGGTT CAAGTGTCGG TATTTCTAAG TCTGAAAACC
S. anginosus    AAAACCGTCA AATATGGGAT CTAGTGTCGG CATTTCTAAG TCTGAAAATC
S. constellatus AAAACCGTCA AATATGGGCT CTAGTGTCGG CATTTCTAAG TCTGAAAATC
S. intermedius  TAAACCATCA AATATGGGTT CTAGTGTTGG CATTTCTAAA TCTGAAAACA
S. salivarius   CAAACCTGCT AATATGGGGT CATCTGTTGG GATTTCTAAA GCTGAAAATG
S. mutans       TAAGCCAGCT AATATGGGTT CTAGTGTTGG TATTTCTAAA GCAGAAAATC
                 ** **  *  ** ***** * *   *** **  ***** **   * **  *

                251                                                300
S. gordonii     AGGAAGAACT GCGTGCTTCT CTGGACCTGG CTTTCAAATA CGATAGTCGG
S. sanguis      AAGCAGAACT GCGTGCTTCT CTGGACCTGG CTTTCAA..T ACGACGCCGA
S. mitis        AAGAGGAACT TCGTCAAGCC TTGAAACTTG CCTTCCAATA TGATAGCCGT
S. oralis       AAGAGGAACT CCGTCAAGCT TTGGAACTTG CCTTCCAATA TGACAGCCGT
S. anginosus    AAGATGAATT GCGCTCTGCT CTTGAATTGG CTTTCAAATA TGATAGCCGT
S. constellatus AAGATGAATT GCGTTCTGCT CTTGAATTGG CTTTCAAATA TGATAGCCGT
S. intermedius  AAGATGAATT GTGTTCTGCT CTTAAGTTAG CTTTTAAATA CGATACCCGT
S. salivarius   AAGCAGAGCT TCGTGCAGCG ATTGATCTGG CTCTCAAATA TGATAGCCGT
S. mutans       GCACTGACTT AAAACAAGCT ATTGCACTTG CTTTGAAGTA TGACAGTCGT
                     **  *         *   *     *   *  *  *           **

                301                                                350
S. gordonii     GTACTAGTCG AGCAAGG... TGTAACAGCT CGTGAGATTG AGGTTGGACT
S. sanguis      GTACTGGTTG AGCAAGGAGA TCGAACGGCT CGCGAGATTG AGGTTGGGCT
S. mitis        GTCTTGGTTG AACAAGG... GGTAAATGCC CGTGAAATCG AGGTTGGCCT
S. oralis       GTCTTGGTAG AGCAAGG... GGTGAATGCC CGTGAAATCG AGGTTGGTCT
S. anginosus    GTCTTGATTG AGCAAGG... TGTCAATGCG CGTGAAATTG AAGTTGGTTT
S. constellatus GTCTTGATTG AGCAAGG... TGTCAATGCG CGTGAAATTG AAGTTGGTTT
S. intermedius  GTCTTGATTG AGCAAGG... TGTCAATGCG CGTGAAATTG AAGTTGGTTT
S. salivarius   ATCTTGATTG AGCAAGG... TGTGGTTGCC CGTGAAATCG AGGTTGGTAT
S. mutans       GTTTTAATTG AACAAGG... TGTGGATGCG CGTGAGATTG AGGTTGGTAT
                 *  *  * * * *****           ** ** ** ** * * *****  *

                351                                                400
S. gordonii     TCTTGGTAAC ACTGATGTCA AAAGCAGTCT TCCAGGGGAA GTTGTTAAGG
S. sanguis      TTTGGGCAAT GTTGACGTCA AGAGTACCCT ACCTGGAGAA GTGGTCAAGG
S. mitis        ACTGGGTAAC TACGATGTTA AAAGCACGCT TCCAGGAGAA GTAGTTAAGG
S. oralis       CTTGGGCAAC TACGATGTGA AGAGCACGCT TCCTGGTGAA GTGGTCAAGG
S. anginosus    ACTTGGAAAT GAAGGAGCCA AAAGCAGTTT GCCAGGTGAG GTAGTGAAAG
S. constellatus ACTTGGAAAT GAAGGAGCCA AAAGCAGTTG GCCAGGTGAG GTAGTGAAAG
S. intermedius  ATTTGGGAAC GAAGGAGCTA AAAGTAGTTT ACCAGGTGAA GTGGTGAAAG
S. salivarius   CCTTGGCAAT ACGACTGTCA AAACGACTAA TCCAGGTGAA GTAGTCAAAG
S. mutans       TTTAGGAAAT ACTGATGTTA AAACAACTTT ACCGGGAGAG ATTGTCAAAG
                  * ** **        * * * *  *      ** ** **   * ** ** *
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                201                                                250
S. gordonii     CAAACCTTCT AATATGGGCT CCAGTGTCGG CATTTCAAAA TCTGATAATC
S. sanguis      CAAACCTTCT AATATGGGCT CCAGTGTCGG CATTTCTAAA TCGGA.GACC
S. mitis        AAAACCATCT AACATGGGTT CTAGTGTCGG TATTTCTAAG TCTGAAAATC
S. oralis       GAAGCCGTCA AACATGGGTT CAAGTGTCGG TATTTCTAAG TCTGAAAACC
S. anginosus    AAAACCGTCA AATATGGGAT CTAGTGTCGG CATTTCTAAG TCTGAAAATC
S. constellatus AAAACCGTCA AATATGGGCT CTAGTGTCGG CATTTCTAAG TCTGAAAATC
S. intermedius  TAAACCATCA AATATGGGTT CTAGTGTTGG CATTTCTAAA TCTGAAAACA
S. salivarius   CAAACCTGCT AATATGGGGT CATCTGTTGG GATTTCTAAA GCTGAAAATG
S. mutans       TAAGCCAGCT AATATGGGTT CTAGTGTTGG TATTTCTAAA GCAGAAAATC
                 ** **  *  ** ***** * *   *** **  ***** **   * **  *

                251                                                300
S. gordonii     AGGAAGAACT GCGTGCTTCT CTGGACCTGG CTTTCAAATA CGATAGTCGG
S. sanguis      AAGCAGAACT GCGTGCTTCT CTGGACCTGG CTTTCAA..T ACGACGCCGA
S. mitis        AAGAGGAACT TCGTCAAGCC TTGAAACTTG CCTTCCAATA TGATAGCCGT
S. oralis       AAGAGGAACT CCGTCAAGCT TTGGAACTTG CCTTCCAATA TGACAGCCGT
S. anginosus    AAGATGAATT GCGCTCTGCT CTTGAATTGG CTTTCAAATA TGATAGCCGT
S. constellatus AAGATGAATT GCGTTCTGCT CTTGAATTGG CTTTCAAATA TGATAGCCGT
S. intermedius  AAGATGAATT GTGTTCTGCT CTTAAGTTAG CTTTTAAATA CGATACCCGT
S. salivarius   AAGCAGAGCT TCGTGCAGCG ATTGATCTGG CTCTCAAATA TGATAGCCGT
S. mutans       GCACTGACTT AAAACAAGCT ATTGCACTTG CTTTGAAGTA TGACAGTCGT
                     **  *         *   *     *   *  *  *           **

                301                                                350
S. gordonii     GTACTAGTCG AGCAAGG... TGTAACAGCT CGTGAGATTG AGGTTGGACT
S. sanguis      GTACTGGTTG AGCAAGGAGA TCGAACGGCT CGCGAGATTG AGGTTGGGCT
S. mitis        GTCTTGGTTG AACAAGG... GGTAAATGCC CGTGAAATCG AGGTTGGCCT
S. oralis       GTCTTGGTAG AGCAAGG... GGTGAATGCC CGTGAAATCG AGGTTGGTCT
S. anginosus    GTCTTGATTG AGCAAGG... TGTCAATGCG CGTGAAATTG AAGTTGGTTT
S. constellatus GTCTTGATTG AGCAAGG... TGTCAATGCG CGTGAAATTG AAGTTGGTTT
S. intermedius  GTCTTGATTG AGCAAGG... TGTCAATGCG CGTGAAATTG AAGTTGGTTT
S. salivarius   ATCTTGATTG AGCAAGG... TGTGGTTGCC CGTGAAATCG AGGTTGGTAT
S. mutans       GTTTTAATTG AACAAGG... TGTGGATGCG CGTGAGATTG AGGTTGGTAT
                 *  *  * * * *****           ** ** ** ** * * *****  *

                351                                                400
S. gordonii     TCTTGGTAAC ACTGATGTCA AAAGCAGTCT TCCAGGGGAA GTTGTTAAGG
S. sanguis      TTTGGGCAAT GTTGACGTCA AGAGTACCCT ACCTGGAGAA GTGGTCAAGG
S. mitis        ACTGGGTAAC TACGATGTTA AAAGCACGCT TCCAGGAGAA GTAGTTAAGG
S. oralis       CTTGGGCAAC TACGATGTGA AGAGCACGCT TCCTGGTGAA GTGGTCAAGG
S. anginosus    ACTTGGAAAT GAAGGAGCCA AAAGCAGTTT GCCAGGTGAG GTAGTGAAAG
S. constellatus ACTTGGAAAT GAAGGAGCCA AAAGCAGTTG GCCAGGTGAG GTAGTGAAAG
S. intermedius  ATTTGGGAAC GAAGGAGCTA AAAGTAGTTT ACCAGGTGAA GTGGTGAAAG
S. salivarius   CCTTGGCAAT ACGACTGTCA AAACGACTAA TCCAGGTGAA GTAGTCAAAG
S. mutans       TTTAGGAAAT ACTGATGTTA AAACAACTTT ACCGGGAGAG ATTGTCAAAG
                  * ** **        * * * *  *      ** ** **   * ** ** *
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FIG. 1. Comparison of the sequences of fragments within genes coding for D-Ala:D-Ala ligases from *S. anginosus* (ATCC 33397), *S. constellatus* (ATCC 27823), *S. gordonii* (ATCC 10558), *S. intermedius* (ATCC 27335), *S. mitis* (NCTC 12261), *S. mutans* (NCTC 10449), *S. oralis* (NCTC 7864), *S. salivarius* (ATCC 9758), and *S. sanguis* (NCTC 7863). Asterisks indicate nucleotides that were identical in all sequences. Dots indicate gaps introduced to optimize alignment.

intermedius ATCC 27335, *S. mitis* NCTC 12261, *S. mutans* NCTC 10449, *S. oralis* NCTC 7864, *S. salivarius* ATCC 9758, and *S. sanguis* NCTC 7863. A total of 91 *Streptococcus* strains from the bioMérieux collection (La Balme-les-Grottes, France) were also tested. The species and number of the 91 strains tested were as follows: *S. anginosus*, 9; *S. constellatus*, 10; *S. gordonii*, 10; *S. intermedius*, 6; *S. mitis*, 10; *S. mutans*, 10; *S. oralis*, 10; *S. salivarius*, 10; *S. sanguis*, 10; and *S. vestibularis*, 6. Plasmid pCRII (Invitrogen, San Diego, Calif.) was used for the cloning of the PCR products. *Escherichia coli* INVαF′ One Shot (Invitrogen) was the host strain for recombinant plasmids. The recombinant plasmids were pAT437 (*ddl* gene from *S. bovis* [$ddl_{S.\ bovis}$]), pAT438 ($ddl_{S.\ gordonii}$), pAT439 ($ddl_{S.\ mitis}$), pAT440 ($ddl_{S.\ mutans}$), pAT441 ($ddl_{S.\ oralis}$), pAT442 ($ddl_{S.\ salivarius}$), pAT443 ($ddl_{S.\ sanguis}$), pAT444 ($ddl_{S.\ anginosus}$), pAT445 ($ddl_{S.\ constellatus}$), and pAT446 ($ddl_{S.\ intermedius}$). All strains were grown at 37°C in brain heart infusion broth and on agar (Difco Laboratories, Detroit, Mich.) not supplemented or supplemented with horse blood (5% [vol/vol]). For milleri group streptococci, the incubation was under anaerobic conditions.

DNA manipulation. Total DNA from streptococci was prepared by the cetyldimethylethyl-ammonium bromide method (1). Degenerate oligodeoxynucleotides V1 [GGIGA(A/G)GA(T/C)GGI(T/A)(C/G)I(T/C/A)TICA(A/G)GG] and V2 [TT(A/G)TGI(T/A/G)AIGGICCIAA(A/G)TG] (10), where I stands for inosine, are complementary to sequences encoding conserved amino acid motifs in D-Ala:D-Ala ligases of *E. coli* (22, 28) and the related glycopeptide resistance enzyme VanA (9). Amplification of DNA fragments by PCR using ca. 50 ng of template DNA and primers V1 and V2 at the concentration of 0.1 μM each in a total volume of 100 μl was performed with a DNA thermal cycler (model 2400; Perkin-Elmer Cetus, Emeryville, Calif.) as described previously (10). The *Taq* DNA polymerase was purchased from Amersham Life Science (Cleveland, Ohio). The PCR conditions were as follows: 2 min at 94°C for the first step; 30 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C; and 10 min at 72°C for the last step. The PCR products were purified by agarose gel electrophoresis followed by extraction from the cut-out low-melting-point agarose block with the Sephaglas Kit (Pharmacia, Uppsala, Sweden). Recombinant plasmids were prepared by the Wizard Miniprep proce-

TABLE 2. Oligodeoxynucleotide primers for the second PCR

Streptococcal group and species	Size of PCR product (bp)	Oligodeoxynucleotide		Positions[a]	GC content (%)
		Pair	Sequence		
Group A or milleri group	217	E	5′-TGCAGAAGTAGAGGCAAATC-3′	162–181	45
			3′-TTCCTCGGTTTTCGTCAACCG-5′	362–382	52
Group B					
S. mitis	259	F	5′-TGAAATCGAGGTTGGCCTAC-3′	333–352	50
			3′-TTCCC(G/T)CTCTAAAAGGATTTGC-5′	571–592	45
S. oralis	563	G	5′-CTTATGTCGGCTGCAATATCC-3′	23–43	47
			3′-TTCCC(G/T)CTCTAAAAGGATTTGC-5′	571–592	45
Group C					
S. gordonii	260	H	5′-GTCGATGGCGAGGATCTAGAGC-3′	133–154	59
			3′-CAGAAGGTCCCCTTCAACAA-5′	377–396	50
S. sanguis	374	I	5′-GTCGATGGCGAGGATCTAGAGC-3′	133–154	59
			3′-GACTACGCAGTTTTACGTCTC-5′	490–510	47

[a] Positions were derived from the alignment in Fig. 1.

dure (Promega, Madison, Wis.). The DNA sequences of PCR products were determined by the dideoxynucleotide chain terminator technique (24) by using universal or specific oligodeoxynucleotides (Unité de Chimie Organique, Institut Pasteur, Paris, France) as the primers, [α-^{35}S]dATP (Amersham Radiochemical Centre, Amersham, England), and T7 DNA polymerase (T7 Sequencing Kit; Pharmacia) according to the manufacturer's recommendations. For Southern hybridization, DNA was transferred by vacuum onto Nytran membranes (Schleicher and Schuell, Dassel, Germany). Prehybridization and hybridization were performed under stringent conditions at 68°C in 0.1% sodium dodecyl sulfate–0.05% nonfat dry milk–6$\times$ SSC (1$\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 3 and 18 h, respectively. Probes were labeled with [α-^{32}P]dCTP (Amersham Radiochemical Centre) by the nick translation procedure (Nick Translation Kit; Amersham International, Little Chalfont, England).

Sequence analysis. Nucleotide sequences were analyzed with Genetics Computer Group software (6), and the phylogenetic analysis was performed with the PHYLIP program package (12).

Nucleotide sequence accession numbers. The sequences were submitted to GenBank and were assigned the following accession numbers: U69162 (*ddl*$_{S. bovis}$), U69163 (*ddl*$_{S. gordonii}$), U69164 (*ddl*$_{S. mitis}$), U69165 (*ddl*$_{S. mutans}$), U69166 (*ddl*$_{S. oralis}$), U69167 (*ddl*$_{S. salivarius}$), U69168 (*ddl*$_{S. sanguis}$), U91912 (*ddl*$_{S. anginosus}$), U91913 (*ddl*$_{S. intermedius}$), and U91914 (*ddl*$_{S. constellatus}$).

RESULTS AND DISCUSSION

Design of oligodeoxynucleotides. Internal portions (ca. 600 bp) of the genes coding for D-Ala:D-Ala ligases in nine species of streptococci (*S. anginosus*, *S. constellatus*, *S. gordonii*, *S. intermedius*, *S. mitis*, *S. mutans*, *S. oralis*, *S. salivarius*, and *S. sanguis*) were amplified by PCR with oligodeoxynucleotides V1 and V2 and cloned into the pCRII vector. Southern hybridization with total DNA of each strain was carried out to confirm the origins of the PCR products (data not shown) that were subsequently sequenced on both strands. Sequence comparison indicated that the inserts corresponded to internal portions of *ddl* genes.

The partial sequences of the nine *ddl* genes were aligned (Fig. 1) and showed high degrees of identity. Pairs of oligodeoxynucleotides, each intended to prime amplification of a fragment within a *ddl* gene, were selected in nonconserved regions.

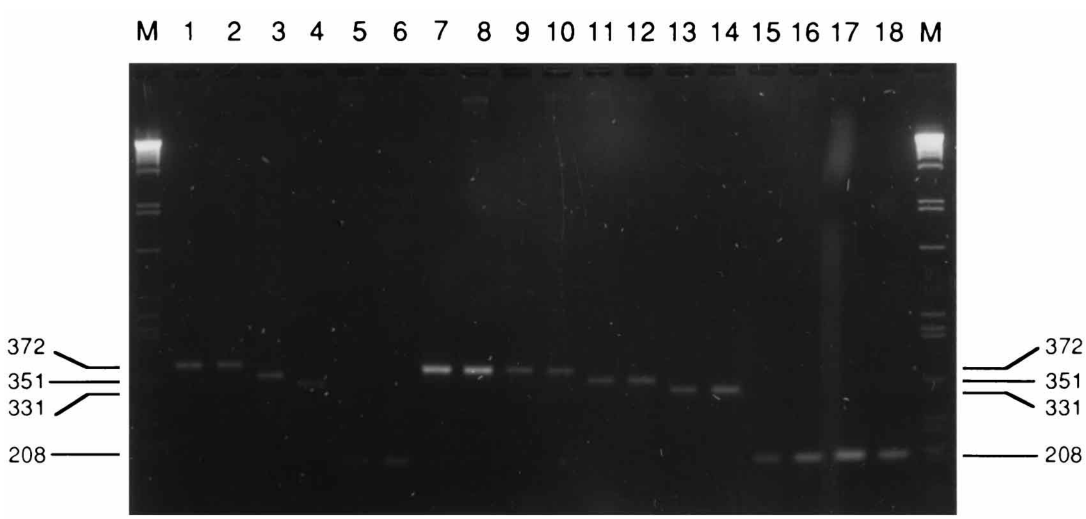

FIG. 2. First-PCR analysis of total DNA from reference and wild strains of viridans group streptococci. Lanes: 1, *S. mitis* NCTC 12261; 2, *S. oralis* NCTC 7864; 3, *S. mutans* NCTC 10449; 4, *S. salivarius* ATCC 9758; 5, *S. gordonii* ATCC 10558; 6, *S. sanguis* NCTC 7863; 7 and 8, *S. mitis*; 9 and 10, *S. oralis*; 11 and 12, *S. mutans*; 13 and 14, *S. salivarius*; 15 and 16, *S. gordonii*; 17 and 18, *S. sanguis*; M, bacteriophage λ DNA (Pharmacia) digested with *Pst*I used as size standards. PCR products were resolved by electrophoresis on a 2% agarose–Tris–borate–EDTA gel containing 0.5 μg of ethidium bromide per ml. The sizes of the PCR products (in base pairs) are indicated to the sides of the gel.

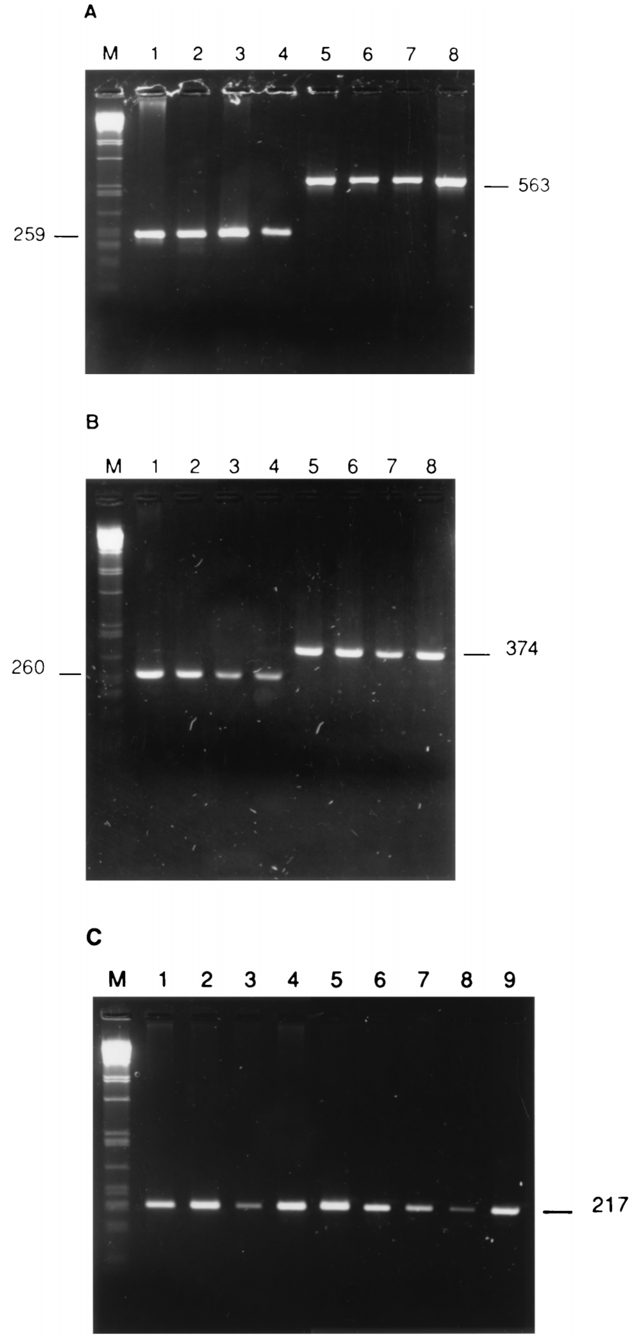

FIG. 3. Second-PCR analysis of total DNA from wild and reference strains of viridans group streptococci. (A) PCR with oligonucleotide pairs F and G. Lanes: 1, *S. mitis* NCTC 12261; 2 to 4, *S. mitis*; 5, *S. oralis* NCTC 7864; 6 to 8, *S. oralis*. (B) PCR with oligonucleotide pairs H and I. Lanes: 1, *S. gordonii* ATCC 10558; 2 to 4, *S. gordonii*; 5, *S. sanguis* NCTC 7863; 6 to 8, *S. sanguis*. (C) PCR with oligonucleotide pair E. Lanes: 1, *S. anginosus* ATCC 33397; 2, *S. constellatus* ATCC 27823; 3, *S. intermedius* ATCC 27335; 4 and 5, *S. anginosus*; 6 and 7, *S. constellatus*; 8 and 9, *S. intermedius*. In all panels, lanes M contained bacteriophage λ DNA (Pharmacia) digested with *Pst*I used as size standards. PCR products were resolved by electrophoresis on a 2% agarose–Tris–borate–EDTA gel containing 0.5 μg of ethidium bromide per ml. The sizes of the PCR products (in base pairs) are indicated to the sides of the gels.

Primers of similar sizes and with a GC content ranging from 43 to 60% were designed to avoid variations in annealing temperature and to allow their simultaneous use in a single reaction mixture. However, due to the small sizes of the internal fragments sequenced (600 bp), the high degrees of identity for the nine sequences, and the fact that each amplification product should be assigned to a species on the basis of its size, a two-stage PCR appeared necessary. In the first PCR with oligodeoxynucleotide pairs A to D, the amplification products obtained with pairs B and C could be assigned to a single species (Table 1). Pair A amplified *S. oralis* and *S. mitis*, and pair D amplified both *S. gordonii* and *S. sanguis*. A second PCR using primers F and G or H and I (Table 2) allowed the differentiation of *S. oralis* from *S. mitis* or *S. gordonii* from *S. sanguis*, respectively, whereas pair E (Table 2), used alone, allowed identification of the species from the milleri group.

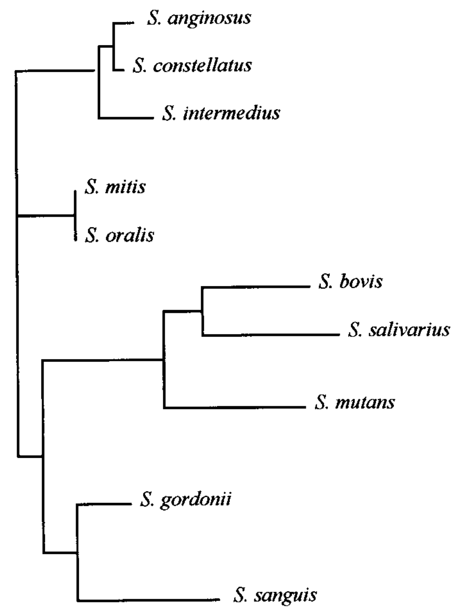

FIG. 4. Phylogenetic relationship among streptococci. The tree was constructed by the neighbor-joining method and slightly modified taking into account the results of maximum-parsimony and bootstrapping analysis.

PCR experiments. PCRs were performed with DNA from every reference strain as a template. Occurrence of nonspecific bands led us to modify the PCR conditions as follows: (i) for the first PCR and for the second PCR with oligonucleotide pairs F and G or H and I, 2 min at 94°C for the first step; 20 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; and 10 min at 72°C for the last step; and (ii) for the second PCR with oligonucleotide pair E, 2 min at 94°C for the first step; 30 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; and 10 min at 72°C for the last step.

The sizes of the amplification products obtained under these conditions differed sufficiently to allow identification of the reference strains (data not shown).

We finally investigated our strategy by testing 91 characterized strains of viridans group streptococci. The PCR results confirmed the identification of 60 strains (10 *S. gordonii*, 10 *S. mitis*, 10 *S. mutans*, 10 *S. oralis*, 10 *S. salivarius*, and 10 *S. sanguis* strains) to the species level (Fig. 2 and 3A and B and data not shown) and the assignment of 25 strains (10 *S. anginosus*, 9 *S. constellatus*, and 6 *S. intermedius* strains) to the milleri group (Fig. 3C and data not shown), whereas the 6 strains of *S. vestibularis* were identified as *S. salivarius* (data not shown). A relatively high degree of relatedness has been observed by DNA-DNA hybridization between strains of *S. vestibularis* and *S. salivarius* (5, 27). These data are consistent with the observation that the oligonucleotides designed for *S. salivarius* also amplified a fragment of total DNA from *S. vestibularis*. However, to the best of our knowledge, *S. vestibularis* has not been reported to be responsible for purulent infections, endocarditis, septicemia, or meningitis and is thus unlikely to be isolated from foci of infection.

Phylogenetic analysis. The amino acid sequence deduced from the DNA region between oligonucleotides V1 and V2 of 10 species (*S. anginosus*, *S. bovis*, *S. constellatus*, *S. gordonii*, *S. intermedius*, *S. mitis*, *S. mutans*, *S. oralis*, *S. salivarius*, and *S. sanguis*) was used for phylogenetic analysis (Fig. 4). The phylogeny obtained was compared with that derived from 16S rRNA sequences (17). The topologies of the two trees obtained with the neighbor-joining method (12) were superimposable except for *S. gordonii* and *S. constellatus.* The difference concerns the position of nodes of these two species.

Identification of viridans group streptococci to the species level is required for certain infections. DNA-DNA hybridization with the type strain is the "gold standard" technique for identification to the species level. However, this method requires radioisotopes and involves complex procedures, and its application is thus limited to research or reference laboratories. Our PCR assay provides a specific and rapid alternative to phenotypic or DNA-DNA hybridization methods for identification of clinically relevant viridans group streptococci to the species or group level within 48 h from the time of isolation of the microorganism.

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