JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2010, p. 3532–3538 Vol. 48, No. 10
0095-1137/10/$12.00 doi:10.1128/JCM.00787-10

Use of *groESL* as a Target for Identification of *Abiotrophia*, *Granulicatella*, and *Gemella* Species▽

Wei-Chun Hung,[1] Sung-Pin Tseng,[1] Hsiao-Jan Chen,[1] Jui-Chang Tsai,[2,4] Chih-Hsin Chang,[3] Tai-Fen Lee,[3] Po-Ren Hsueh,[3] and Lee-Jene Teng[1,3]*

Department of Clinical Laboratory Sciences and Medical Biotechnology,[1] Center for Optoelectronic Biomedicine,[2] National Taiwan University College of Medicine, Department of Laboratory Medicine,[3] and Division of Neurosurgery, Department of Surgery,[4] National Taiwan University Hospital, Taipei, Taiwan

Received 19 April 2010/Returned for modification 8 July 2010/Accepted 27 July 2010

We determined the *groESL* sequences of three species of nutritionally variant streptococci (*Abiotrophia defectiva*, *Granulicatella adiacens*, and *Granulicatella elegans*) and three *Gemella* species (*Gemella morbillorum*, *Gemella haemolysans*, and *Gemella sanguinis*). The nucleotide sequence similarities between the *groES* and *groEL* genes of the above genera were 41.7 to 85.9% and 63.7 to 84.3%, respectively. The intraspecies similarities of *groESL* sequences for the isolates of *Abiotrophia* and *Granulicatella* species were 94.4 to 97.8% for *groES* and 94.0 to 98.2% for *groEL*. For *Ge. morbillorum* and *Ge. sanguinis*, all strains showed the same *groESL* spacer length (8 bp), and sequence identities within species were >97.8% for *groES* and >96.1% for *groEL*. However, higher intraspecies heterogeneity was observed in *Ge. haemolysans*. Phylogenetic analysis of *groEL* sequences separated the 6 isolates of *Ge. haemolysans* into two subgroups. Among these isolates, three isolates with the same *groESL* spacer region length (45 bp) clustered together but were distant from the ATCC reference strain (with a spacer length of 8 bp). The remaining three isolates, with a spacer length of 50 or 8 bp, clustered together. Although 16S rRNA gene sequence analysis did not provide enough discrimination for the 6 *Ge. haemolysans* isolates, *rpoB* gene sequence analysis supported the subgrouping. Based on the obtained *groESL* sequences, we developed a multiplex PCR that enables simple, rapid, and accurate identification of *Abiotrophia*, *Granulicatella*, and *Gemella* at the genus level. This assay would be helpful for identifying these fastidious and slow-growing organisms in clinical laboratories.

Abiotrophia, *Granulicatella*, and *Gemella* species are major fastidious, Gram-positive cocci that cause infective endocarditis and can easily be misidentified or even nonculturable (1).

Abiotrophia and *Granulicatella* species were previously known as nutritionally variant streptococci (NVS). Among NVS, the most common species causing human disease are *Abiotrophia defectiva*, *Granulicatella adiacens*, and *Granulicatella elegans*. As a normal part of the floras of the mouth and gastrointestinal tract, NVS can opportunistically cause sepsis, pancreatic abscess, central nervous system infections, and endocarditis (7, 22, 28). NVS have reportedly been implicated in a case of culture-negative endocarditis (21), so their pathogenic roles are probably underestimated.

Gemella haemolysans and *Gemella morbillorum* are part of the oral, gastrointestinal, and genitourinary floras of humans. These species are easily decolorized in Gram stains, which can lead to their misidentification as Gram-negative cocci. *Gemella* was originally described as *Neisseria* species in 1938 (26). Among cases of *Gemella* infections, the most notable clinical presentation is endocarditis, which is usually associated with poor dental conditions or previously damaged cardiac valves (1, 17). In the clinical laboratory, misidentification of *Gemella* species as viridans group streptococci occurs quite often. In previous studies, *Gemella* species represented 6% to 13.3% of misidentified viridans group streptococci (5, 17).

Differentiation of NVS, *Gemella*, and other Gram-positive cocci is clinically important, partly because the antimicrobial susceptibility patterns of these bacteria are different. In Taiwan, the *Gemella* species remain susceptible to penicillin and metronidazole (16), while NVS isolates have a high prevalence of β-lactam and macrolide resistance (18).

The current identification methods for NVS and *Gemella* are based on phenotypic characterization, which is time-consuming and can produce ambiguous results. In addition, unusual phenotypic characteristics that do not correlate well with those of the NVS species described in the literature have been reported (3). The accuracy of commercial kits commonly used for clinical identification was evaluated by Woo et al. (30), and their results showed frequent misidentification of *Gemella* species as *Streptococcus*, *Abiotrophia*, or *Granulicatella* species. Recently, several molecular methods have been developed to simultaneously identify NVS and *Gemella*; these methods include *rpoB* gene sequence-based identification (4) and the MicroSeq 500 16S rRNA gene-based bacterial identification system (31).

The *groES* and *groEL* genes are ubiquitous and evolutionarily highly conserved genes among bacteria (11). They are also known as *cpn10/60* or *hsp10/60*, encoding 10-kDa and 60-kDa heat shock proteins responsible for refolding denatured proteins or preventing aggregation while bacteria are exposed to elevated temperature or other stress. A collection of the chaperonin sequence database (cpnDB) is available online for public use (13). Amplification of the partial *groEL* gene segment

* Corresponding author. Mailing address: Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, 1 Chang-Te Street, Taipei 100, Taiwan. Phone: 886-2-23123456, ext. 66918. Fax: 886-2-23711574. E-mail: ljteng@ntu.edu.tw.

▽ Published ahead of print on 4 August 2010.

TABLE 1. Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Gene	Nucleotide positions
Universal amplification primers			
Gor600F	GGNGAYGGNACNACNACNGCNACNGT	*groEL*	253–278
Gor600R	TCNCCRAANCCNGGYGCNTTNACNGC	*groEL*	842–817
LA PCR amplification primers			
C1	GTACATATTGTCGTTAGAACGCGTAATACGACTCA		
C2	CGTTAGAACGCGTAATACGACTCACTATAGGGAGA		
LA-Gm-F1	GTTGAACGTCTTCGTGAAATTAGCGTAAC	*groEL*	377–405
LA-Gm-F2	AAGTAGGTGCTATTTCTGCTGCGGATGAA	*groEL*	432–460
LA-Gm-R1	GTCGCTATAGCTTCTTGTTCGACATCATC	*groEL*	774–746
LA-Gm-R2	GCAGCAGCAACTACTTCTTCTAAAACTGG	*groEL*	723–695
Primers for PCR amplification of *Gemella* species			
Gm-400F	CTTCATATCCGAAGTGCGATGTT	*groES*	−398–−420
ORF179-199R	CTCCGTATTCTCCAGCTGCTT	*trxA*	199–179
Primers for PCR amplification of NVS			
Ent-ES-UP	TTATAAAYAGTGTRRGTTAGCACTC		
EL587-563R	TADCCNCGRTCAAATTGCATHCCTT	*groEL*	587–563
ES5-29F	TAAAACCHTTAGGHGAHCGWRTBGT	*groES*	5–29
EL1240R	CCACCACCWGVWACNAWDCCTTC	*groEL*	1241–1219
Primers for multiplex PCR			
Abio EL220F	GTGCTAGAAGTGGCTAG	*groEL*	217–233
Gra ES200F	CACTGAAGTTAAATTGGATGGTC	*groES*	198–220
Gem EL30F	CAGAAGATGCTCGCCAAT	*groEL*	23–40
NG EL570R	CGRTCAAATTGCATHCCTTC	*groEL*	584–565

[a] Y = T or C, H = A, T, or C, R = A or G, N = A, T, G, or C, W = A or T, B = T, G, or C, V = A, G, or C, and D = A, T, or G.

has been used for identification of many bacteria. In our laboratory, we previously used *groESL* gene sequences as targets for *Enterococcus* (25, 27) and *Streptococcus* (2, 14, 19, 24) species identification. In this study, we determined the full *groESL* gene sequence for 3 reference strains of *Gemella* species and partial *groESL* sequences for reference strains of *Abiotrophia defectiva*, *Granulicatella adiacens*, and *Granulicatella elegans*. Based on the *groESL* sequences determined, we developed a multiplex PCR for rapid identification of the fastidious genera *Abiotrophia*, *Granulicatella*, and *Gemella*.

MATERIALS AND METHODS

Bacterial strains. The strains examined in this study include 6 reference strains and 30 clinical isolates. The reference strains were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and included *A. defectiva* ATCC 49176, *Gr. adiacens* ATCC 49175, *Gr. elegans* ATCC 700633, *Ge. morbillorum* ATCC 27824, *Ge. haemolysans* ATCC 10379, and *Gemella sanguinis* ATCC 700632. The clinical isolates were collected between 1997 and 2009 in the Bacteriology Laboratory, National Taiwan University Hospital (NTUH), which is a 2,500-bed teaching hospital in northern Taiwan. The clinical isolates, including 7 *A. defectiva*, 10 *Gr. adiacens*, 1 *Granulicatella para-adiacens*, 1 *Gr. elegans*, 2 *Ge. morbillorum*, 4 *Ge. sanguinis*, and 5 *Ge. haemolysans* strains, were identified by commercial identification systems (Vitek system, API 20 Strep system [bioMerieux Vitek], Phoenix system, and Rapid ID 32 Strep system [bioMerieux Vitek]) and confirmed by 16S rRNA gene sequencing.

Sequencing of the entire *groESL* locus in *Ge. morbillorum*. Genomic DNA was isolated and purified with a DNA isolation kit (Puregene; Gentra Systems) according to the manufacturer's instructions. Initially, degenerate PCR primers complementary to highly conserved regions of the *groEL* gene (Table 1) were designed and used to amplify and sequence partial fragments of this region from *Ge. morbillorum* ATCC 27824. Subsequently, the flanking sequences were determined by long and accurate (LA) PCR as described previously (25). Briefly, the digoxigenin-labeled *groEL* amplicon was used as a probe to determine the most suitable restriction enzyme for LA PCR. After ligating the restriction-digested DNA fragments with the corresponding cassette adapters, the amplification was performed with cassette primers and target-specific primers (Table 1). The amplification products were subsequently sequenced on an Applied Biosystem model 3100 sequencing system (Applied Biosystems), using a *Taq* BigDye Terminator dideoxy cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

PCR amplification and sequencing of *groESL* for other *Gemella* species. Two pairs of primers, Gm-400F with LA-Gm-R2 and LA-Gm-F2 with ORF179-199R, were used to amplify two overlapping fragments and to determine the complete *groESL* sequences of *Gemella* species. PCR was carried out in a DNA thermal cycler (MJ Research Inc., Watertown, MA), with 30 cycles of denaturation (94°C, 30 s), annealing (50 to 60°C, 1 min), and extension (72°C, 1 min 30 s) followed by a final extension step (72°C, 7 min). The PCR products were purified and subsequently sequenced as mentioned earlier.

PCR amplification and sequencing of *groESL* for *Abiotrophia* and *Granulicatella* species. To determine the complete *groES* and partial *groEL* gene sequences of *Abiotrophia* and *Granulicatella* species, two sets of PCR primers, Ent-10 with EL587-563R and ES5-29F with EL1240R, were used to amplify two overlapping fragments of the *groESL* genes and to determine the sequences of the *groES* gene, the *groESL* spacer region, and an approximately 1,240-bp fragment of the *groEL* gene. PCR was carried out in a DNA thermal cycler (MJ Research Inc., Watertown, MA), with 30 cycles of denaturation (94°C, 30 s), annealing (45 to 55°C, 1 min), and extension (72°C, 1 min 30 s) followed by a final extension step (72°C, 7 min). The PCR products were purified and subsequently sequenced as described above.

Multiplex PCR. Based on the determined sequences, one reverse primer, NG570R, and three forward primers, AbioEL220F, GraES200F, and GemEL30F, specific to *Abiotrophia*, *Granulicatella*, and *Gemella* species, respectively, were designed to amplify a target region with a different amplicon size depending on the species. The multiplex PCR was carried out with 30 cycles of denaturation (94°C, 30 s), annealing (53°C, 30 s), and extension (72°C, 1 min) followed by a final extension step (72°C, 7 min). The amplification products were then subjected to 1.5% agarose gel electrophoresis (FMC BioProducts, Rockland, ME), stained with ethidium bromide, and photographed under UV light.

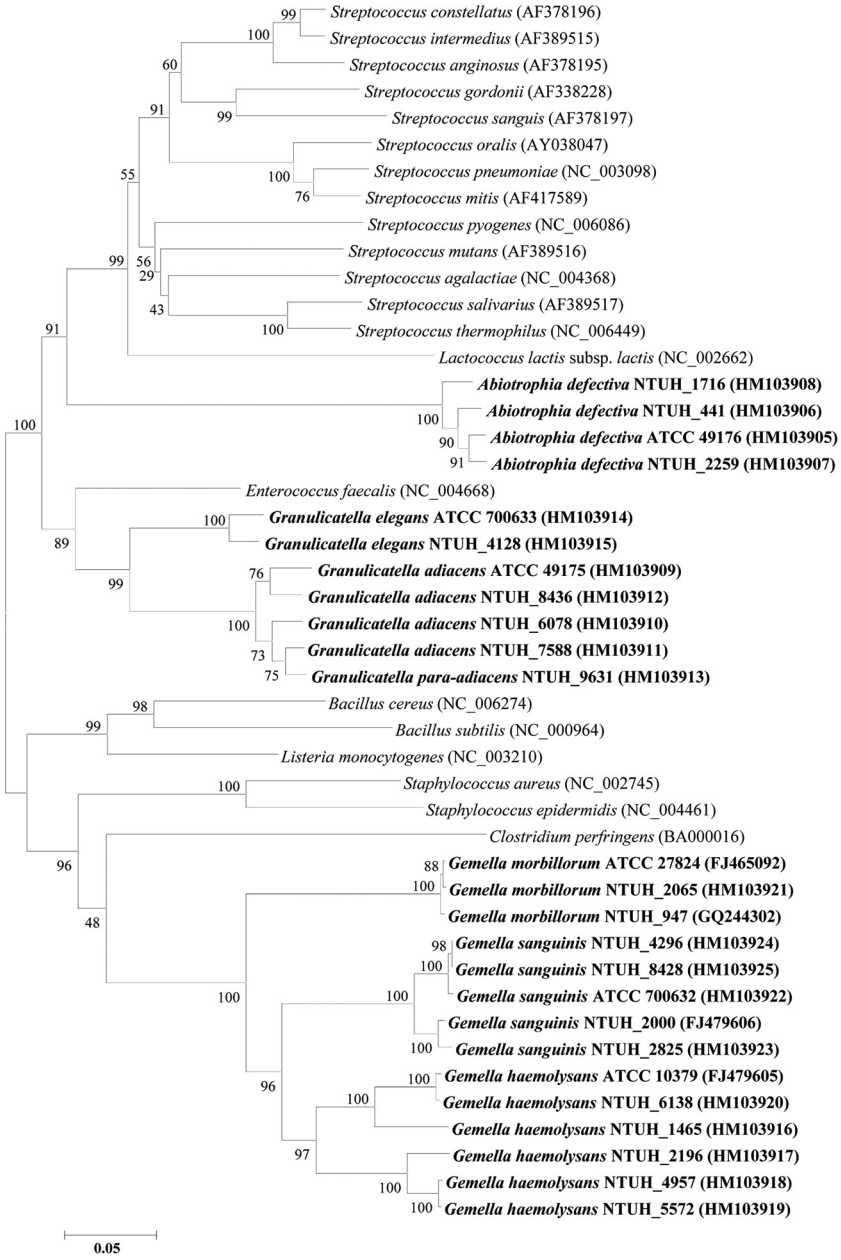

FIG. 1. Phylogenetic tree based on partial *groEL* nucleotide sequences (nt 1 to 816). The phylogenetic tree was generated using the unrooted neighbor-joining method in the MEGA4 package. The numbers at the nodes are confidence levels, expressed as percentages of occurrence in 500 bootstrapped resamplings. The scale bar indicates the evolutionary distance between sequences, as determined by measuring the lengths of the horizontal lines connecting two organisms. The GenBank accession number for each species' *groEL* genes is shown after the species name. Sequences determined in this study are shown in bold.

Phylogenetic relationships. DNA sequences were aligned using Gene-Works software (IntelliGenetics, Mountain View, CA). The phylogenetic relationships among species were analyzed using the neighbor-joining method listed in the MEGA (molecular evolutionary genetic analysis) analytical package (23). For the neighbor-joining analysis, distances between the sequences were calculated using Kimura's two-parameter model. Levels of similarity were determined among species. Bootstrap values were obtained for 500 randomly generated trees.

Nucleotide sequence accession numbers. For *groESL*, nucleotide sequences determined in this study were submitted to GenBank under the accession numbers shown in Fig. 1. For *rpoB*, nucleotide sequences were submitted under GenBank accession numbers HM103926 to HM103930, and for the 16S rRNA gene, sequences were submitted under GenBank accession numbers HM103931 to HM103935.

RESULTS

Nucleotide sequences of *groESL* in *Abiotrophia*, *Granulicatella*, and *Gemella* species. For *Abiotrophia* and *Granulicatella* species, we determined the full-length *groES* gene sequences and the partial *groEL* sequences of 3 reference species and 8 clinical isolates. For *Gemella* species, we first determined the

TABLE 2. *groES* nucleotide and amino acid sequence similarities

Strain no.	Strain	Length of *groES* (bp)	% *groES* sequence similarity[a] with strain:					
			1	2	3	4	5	6
1	*A. defectiva* ATCC 49176	267		56.7	63.4	45.7	41.7	44.7
2	*Gr. adiacens* ATCC 49175	270	68.5		79.3	55.0	54.6	50.9
3	*Gr. elegans* ATCC 700633	270	68.5	93.3		60.2	52.7	56.8
4	*Ge. haemolysans* ATCC 10379	276	53.5	58.5	60.6		80.8	85.9
5	*Ge. morbillorum* ATCC 27824	276	55.7	59.6	60.6	93.4		80.1
6	*Ge. sanguinis* ATCC 700632	276	56.2	57.4	60.6	95.6	92.3	

[a] Data in the upper right portion of the table body indicate nucleotide sequence similarities, and data in the lower left portion indicate amino acid sequence similarities.

complete sequence of the *groESL* region of *Ge. morbillorum* ATCC 27824 by using the LA PCR method. Based on the determined sequence for *Ge. morbillorum* ATCC 27824, two pairs of primers were designed and used to amplify the *groESL* fragment from 13 *Gemella* strains. Sequence analysis revealed that the lengths of the *groES* genes were genus specific and were as follows: for *Abiotrophia*, 267 bp; for *Granulicatella*, 270 bp; and for *Gemella*, 276 bp (Table 2). The length of the *Gemella groEL* gene was 1,605 bp.

Comparisons of *groES*, spacer, and *groEL* sequences among reference strains. Table 2 lists the pairwise nucleotide and amino acid identities of *groES* sequences from 6 reference strains of *Abiotrophia*, *Granulicatella*, and *Gemella*. Comparing bacterial strains among different genera, the *groES* genes displayed nucleotide sequence identities of <63.4% and amino acid sequence identities of <68.5%. The *groES* sequence identities among different species within the same genus ranged from 79.3 to 85.9% (DNA sequences) and 92.3 to 95.6% (amino acid sequences).

Table 3 presents the pairwise sequence identities of the partial *groEL* genes (nucleotides [nt] 1 to 816). The *groEL* nucleotide sequence identities were >81.5% for strains within the same genus and <73.8% for strains from different genera, whereas the amino acid sequence similarities were >98.5% for strains from the same genus and <91.2% for strains from different genera.

The intergenic spacers between the *groES* and *groEL* regions of 6 ATCC reference strains ranged from 8 to 35 nt long (Table 4). The spacers in the three *Gemella* reference strains (*Ge. morbillorum* ATCC 27824, *Ge. haemolysans* ATCC 10379, and *Ge. sanguinis* ATCC 700632) displayed the same size (8 bp), with only 2 nucleotide differences.

Phylogenetic relationships. The phylogenetic tree derived from the nucleotide sequences of the partial *groEL* sequences (positions 1 to 816) is presented in Fig. 1. The phylogenetic analysis revealed that isolates of the same species clustered together. However, the six strains of *Ge. haemolysans* could be divided further, into two subgroups. The NTUH_1465 and NTUH_6138 strains were more closely related to the *Ge. haemolysans* reference strain. The remaining three isolates (NTUH_2196, NTUH_4957, and NTUH_5572) clustered together and were relatively distant from ATCC 10379.

Intraspecies variation in *groESL* sequences of clinical isolates. In order to evaluate the applicability of the *groESL* sequence to species identification, the *groES* sequence, the partial *groEL* sequence, and the *groESL* spacer region of 19 clinical isolates (as indicated in Table 5) were tested to identify intraspecies similarities. Comparisons of species nucleotide identities with the reference strains are shown in Table 5. Most isolates within a species shared >94% sequence identity with their corresponding type strain, except for *Ge. haemolysans*, which shared only 87.0 to 99.5% sequence identity with its type strain.

***groESL*, 16S rRNA, and *rpoB* gene analyses of *Ge. haemolysans* clinical isolates.** Because high intraspecies variation in *groESL* was observed in the *Ge. haemolysans* isolates, we further compared their *rpoB* and 16S rRNA gene nucleotide sequences (Table 6). One *Ge. haemolysans* isolate, NTUH_6138, which had a *groESL* intergenic spacer of identical size (8 bp) and sequence to that of the reference strain, displayed the highest sequence identities for *groES* (99.3%) and *groEL* (99.5%). Isolate NTUH_1465, with a *groESL* spacer region of 50 bp (Table 4), displayed sequence identities of 94.9% (for *groES*) and 92.9% (for *groEL*) to the ATCC strain. Phyloge-

TABLE 3. Partial *groEL* (nt 1 to 816) nucleotide and amino acid sequence similarities

Strain no.	Strain	% *groEL* sequence similarity[a] with strain:					
		1	2	3	4	5	6
1	*A. defectiva* ATCC 49176		70.7	71.1	63.8	63.7	64.0
2	*Gr. adiacens* ATCC 49175	91.2		84.3	67.5	69.3	70.0
3	*Gr. elegans* ATCC 700633	90.8	98.9		73.6	72.0	73.8
4	*Ge. haemolysans* ATCC 10379	85.3	87.1	87.5		81.7	84.3
5	*Ge. morbillorum* ATCC 27824	84.6	87.5	87.9	98.5		81.5
6	*Ge. sanguinis* ATCC 700632	84.6	88.6	89.0	98.9	98.9	

[a] Data in the upper right portion of the table body indicate nucleotide sequence similarities, and data in the lower left portion indicate amino acid sequence similarities.

TABLE 4. Nucleotide sequences and lengths of spacers between *groES* and *groEL* among species

Strain	Spacer length (bp)	Spacer sequence (5′-3′)
A. defectiva ATCC 49176	35	GCACAAGGCAGAGAATAGAAAAGGAGTGACTCTTG
Gr. adiacens ATCC 49175	14	AGGAGGATATCACA
Gr. elegans ATCC 700633	19	GAAAGCGAGGAAATGAAAA
Ge. haemolysans ATCC 10379	8	GAGGAAAC
Ge. haemolysans NTUH_1465	50	TTTTATTACTATTTGGAACAAACAAATAATAAAGAGATTTAGGAGGAAAC
Ge. haemolysans NTUH_2196	45	GTTCGTATCTAAAAAAAGAGATGTTTTAGTATTATAGGAGGAATC
Ge. morbillorum ATCC 27824	8	GAGGATAT
Ge. sanguinis ATCC 700632	8	GAGGAAAT

netic analysis of the partial *groEL* nucleotide sequence (nt 1 to 816) revealed that NTUH_1465 and NTUH_6138 clustered together with the reference strain (Fig. 1). The remaining three isolates, with a 45-bp length for the *groESL* spacer region (Table 4), were >10% different in *groESL* sequence from the reference strain and clustered together. Interestingly, the extra sequences in the four isolates with longer *groESL* spacer lengths (45 or 50 bp) immediately preceded the *groES* stop codon. The extra sequence contains a stop codon at the 5′ end; therefore, the *groES* stop codon of the four clinical isolates changed from TAG to TAA.

The 16S rRNA gene sequences of the five clinical *Ge. haemolysans* isolates were all similar (99.6 to 100%) to that of *Ge. haemolysans* ATCC 10379 (GenBank accession number NR_025903), as shown in Table 6.

Because the *rpoB* gene has been used to identify many species, we performed *rpoB* gene sequencing for the *Ge. haemolysans* clinical isolates. The isolate NTUH_6138, which has the same spacer size (8 bp) as the ATCC 10379 strain, possessed an identical *rpoB* sequence (100%) to that of ATCC 10379 (GenBank accession number AF535179). The *rpoB* genes in the remaining four isolates displayed 95.3 to 97.1% identities to that of ATCC 10379.

Identification of *Abiotrophia*, *Granulicatella*, and *Gemella* by multiplex PCR. Based on the *groESL* sequences obtained in this study, a multiplex PCR method was designed to detect and differentiate the three fastidious Gram-positive genera *Abiotrophia*, *Granulicatella*, and *Gemella*. We designed a degenerate reverse primer, NG570R, and three forward primers, AbioEL220F, GraES200F, and GemEL30F, specific to *Abiotrophia*, *Granulicatella*, and *Gemella*, respectively. The expected sizes of the amplicons were 368 bp for *A. defectiva*, 668 bp for *Gr. adiacens*, 673 bp for *Gr. elegans*, and 562 bp for *Gemella* species. All of the isolates yielded the expected amplicon sizes (some of the data are shown in Fig. 2A).

The specificity of the multiplex PCR method was tested with 14 other commonly encountered Gram-positive bacteria, including *Streptococcus constellatus* ATCC 27823, *Streptococcus intermedius* ATCC 27335, *Streptococcus anginosus* ATCC 33397, *Streptococcus gordonii* CAPQ-2, *Streptococcus sanguinis* ATCC 10556, *Streptococcus oralis* ATCC 35037, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus mitis* ATCC 49456, *Streptococcus pyogenes* ATCC 19615, *Streptococcus agalactiae* ATCC 13813, *Streptococcus salivarius* ATCC 7073, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecium* ATCC 19434, and *Enterococcus faecalis* ATCC 19433. None of these bacterial species generated products of similar size to those for *Abiotrophia*, *Granulicatella*, and *Gemella* (Fig. 2B). Input DNA containing mixed culture organisms did not affect the specific amplification of *Abiotrophia*, *Granulicatella*, and *Gemella*. To determine the sensitivity of detection, PCR with serial dilutions of template DNA was performed. The limit of detection for input DNA is about 2 ng.

DISCUSSION

Application of *groESL* to molecular identification has been evaluated for many bacterial species, including *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Campylobacter*, *Aeromonas*, and others (2, 8–10, 12, 14, 15, 19, 20, 24, 25, 27). In a study by Glazunova et al., the results indicated that *groEL* is a better target than *rpoB*, *sodA*, and *gyrB* for the identification of *Streptococcus* (7a). In the present study, we focused on determining the *groES*, spacer, and *groEL* sequences of 3 species of NVS

TABLE 5. *groESL* identities among clinical isolates, using ATCC strains as references

Species (no. of isolates)	*groES*		Spacer		Partial *groEL* genes (nt 1 to 816)	
	No. of different nucleotides	% Identity	No. of different nucleotides	Length (bp)	No. of different nucleotides	% Identity
A. defectiva (3)	10–15	94.4–96.3	0–1	35–36	15–29	96.5–98.2
Gr. adiacens (4)[a]	8–14	94.8–97.0	3–5	14	33–49	94.0–96.0
Gr. elegans (1)	6	97.8	0	19	28	96.6
Ge. morbillorum (2)	0–4	98.6–100	0	8	2–14	99.5–99.7
Ge. sanguinis (4)	0–6	97.8–100	0	8	4–32	96.1–99.5
Ge. haemolysans (5)	2–29	89.5–99.3	NA[b]	8, 45, or 50	4–106	87.0–99.5

[a] Including one *Gr. para-adiacens* clinical isolate, NTUH_9631.
[b] Not analyzed.

TABLE 6. *groESL*, 16S rRNA, and *rpoB* gene sequence variation of clinical *Ge. haemolysans* strains compared with the reference strain ATCC 10379

Ge. haemolysans isolate	Length of spacer (bp)	% Identity (no. of different nucleotides)			
		groES	*groEL* nt 1 to 816	16S rRNA gene[a]	*rpoB*[b]
NTUH_1465	50	94.9 (14)	92.9 (58)	99.6 (5)	97.1 (18)
NTUH_2196	45	89.5 (29)	87.0 (106)	99.6 (6)	95.3 (29)
NTUH_4957	45	90.9 (25)	87.5 (102)	99.9 (2)	95.6 (27)
NTUH_5572	45	90.9 (25)	87.3 (104)	99.9 (2)	95.6 (27)
NTUH_6138	8	99.3 (2)	99.5 (4)	100 (0)	100 (0)

[a] The length of the 16S rRNA gene sequence determined was 1,364 bp.
[b] The length of the *rpoB* gene sequence determined was 610 bp.

(*A. defectiva*, *Gr. adiacens*, and *Gr. elegans*) and 3 *Gemella* species (*Ge. morbillorum*, *Ge. haemolysans*, and *Ge. sanguinis*). We obtained the *groES* and spacer sequences for all of the species tested. For *groEL*, full-length sequences were obtained for three *Gemella* species, and sequences of nearly 1.2 kb were determined for *Abiotrophia* and *Granulicatella* species. Pairwise comparisons of the *groES* and *groEL* sequences between species revealed more interspecies variation than did comparisons between 16S rRNA and *rpoB* genes (Table 6). The phylogenetic tree derived from the *groEL* gene (Fig. 1) demonstrates remarkable similarities to the tree based on the 16S rRNA gene (6). The tree also reveals that *Abiotrophia* species are related to streptococci and *Gemella* species are most closely related to *Clostridium* and *Staphylococcus* species, whereas *Granulicatella* species are relatively close to *Enterococcus* species.

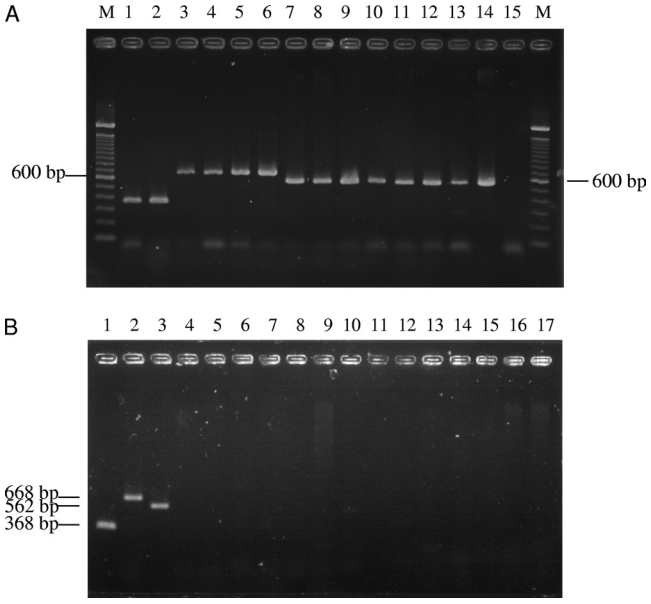

FIG. 2. Multiplex PCR analysis. (A) Multiplex PCR amplification of genomic DNAs of *Abiotrophia*, *Granulicatella*, and *Gemella* species. Lanes M, DNA size markers (100-bp ladder; Invitrogen); lane 1, *A. defectiva* ATCC 49176; lane 2, *A. defectiva* NTUH_9251; lane 3, *Gr. adiacens* ATCC 49175; lane 4, *Gr. adiacens* NTUH_8436; lane 5, *Gr. elegans* ATCC 700633; lane 6, *Gr. elegans* NTUH_4128; lane 7, *Ge. morbillorum* ATCC 27824; lane 8, *Ge. morbillorum* NTUH_2065; lane 9, *Ge. haemolysans* ATCC 10379; lanes 10 to 12, *Ge. haemolysans* NTUH_1465, -2196, and -4957; lane 13, *Ge. sanguinis* ATCC 700632; lane 14, *Ge. sanguinis* NTUH_2000; and lane 15, distilled water as a negative control. (B) Specificity of multiplex PCR tested with 14 other clinically relevant Gram-positive cocci. Lane 1, *A. defectiva* ATCC 49176; lane 2, *Gr. adiacens* ATCC 49175; lane 3, *Ge. morbillorum* ATCC 27824; lane 4, *Streptococcus constellatus* ATCC 27823; lane 5, *Streptococcus intermedius* ATCC 27335; lane 6, *Streptococcus anginosus* ATCC 33397; lane 7, *Streptococcus gordonii* CAPQ-2; lane 8, *Streptococcus sanguinis* ATCC 10556; lane 9, *Streptococcus oralis* ATCC 35037; lane 10, *Streptococcus pneumoniae* ATCC 49619; lane 11, *Streptococcus mitis* ATCC 49456; lane 12, *Streptococcus pyogenes* ATCC 19615; lane 13, *Streptococcus agalactiae* ATCC 13813; lane 14, *Streptococcus salivarius* ATCC 7073; lane 15, *Staphylococcus aureus* ATCC 29213; lane 16, *Enterococcus faecium* ATCC 19434; and lane 17, *Enterococcus faecalis* ATCC 19433.

Previous studies have shown low levels of intraspecies genetic variation between *groES* and *groEL* genes in *Enterococcus* and *Streptococcus* species (24, 27). In agreement with previous reports, the species tested in the present study (except for *Ge. haemolysans*) displayed high sequence identities (>94%) in *groES* and *groEL* gene sequences (Table 5).

Compared to the other species tested, *Ge. haemolysans* displayed more heterogeneity in the *groES*, spacer, and *groEL* sequences. We observed three different lengths for the *groESL* spacer region (8, 45, and 50 bp) within five clinical isolates of *Ge. haemolysans*. One isolate, which had an identical *groESL* spacer sequence (8 bp) to that of the reference strain (*Ge. haemolysans* ATCC 10379), displayed the highest *groES* (99.3%) and *groEL* (99.5%) sequence identities toward the reference strain. Isolates NTUH_2196, NTUH_4957, and NTUH_5572 (with a 45-bp *groESL* spacer length) had the lowest sequence identities of the *groES* (89.5 to 90.9%) and *groEL* (87.0 to 87.5%) regions to the reference strain. Our previous reports have shown that the spacer length between *groES* and *groEL* usually varies among different species but is conserved within a species (14, 24, 27). However, some exceptions occur. For example, minor variations have been found in *Streptococcus gallolyticus*, *S. gallolyticus* subsp. *gallolyticus*, and *S. gallolyticus* subsp. *pasteurianus* (2). In addition to the heterogeneity within the spacer region, the phylogenetic tree based on the *groEL* sequence further divided *Ge. haemolysans* into two subgroups (Fig. 1). Thus, the possibility of a subspecies within *Ge. haemolysans* should be considered. In agreement with the *groEL* analysis, the *rpoB* gene sequence analysis also divided *Ge. haemolysans* into two groups. For 16S rRNA gene sequences, although the levels of similarity were high (99.6 to 100%) among the 6 *Ge. haemolysans* strains, two nucleotide differences (positions 76 and 77) were observed. The nucleotides CG and TA (positions 76 and 77) correlate with spacer lengths of 8 nt and 50 or 45 nt, respectively. This difference

may represent an additional marker for use in subgrouping *Ge. haemolysans* strains.

Misidentification of *Abiotrophia*, *Granulicatella*, and *Gemella* as alpha-hemolytic streptococci may occur. In studies by Woo et al. (29, 30), among 302 isolates of alpha-hemolytic streptococci other than *Streptococcus pneumoniae*, obtained from blood cultures and identified by conventional biochemical methods, 11 isolates had >99% nucleotide identity with the 16S rRNA gene of *A. defectiva* ($n = 2$), *Gr. adiacens* ($n = 7$), *Ge. morbillorum* ($n = 1$), or *Ge. haemolysans* ($n = 1$). Because we did not check our collection of alpha-hemolytic streptococci, the possibility of the above misidentification cannot be excluded. In our collection, one *Gr. para-adiacens* isolate was misidentified as *Ge. morbillorum*. This isolate can be identified correctly to the genus level by using the multiplex PCR developed in this study.

In this study, we developed a multiplex PCR, using *groESL* as a target, for rapid detection of and differentiation among *Abiotrophia*, *Granulicatella*, and *Gemella* isolates. These species phenotypically resemble viridans group streptococci, making their identification difficult. Although this assay can differentiate these species only to the genus level, it is invaluable for differentiating them from *Streptococcus* and other related Gram-positive cocci. The multiplex PCR method will help to decrease the rate of misidentification of these organisms. Moreover, multiplex PCR may be used for direct detection of specimens without cultivation.

ACKNOWLEDGMENT

This work was supported by grant NSC 97-2320-B-002-023-MY3 from the National Science Council of Taiwan.

REFERENCES

1. **Brouqui, P., and D. Raoult.** 2001. Endocarditis due to rare and fastidious bacteria. Clin. Microbiol. Rev. **14:**177–207.
2. **Chen, H. J., J. C. Tsai, T. C. Chang, W. C. Hung, S. P. Tseng, P. R. Hsueh, and L. J. Teng.** 2008. PCR-RFLP assay for species and subspecies differentiation of the *Streptococcus bovis* group based on *groESL* sequences. J. Med. Microbiol. **57:**432–438.
3. **Christensen, J. J., and R. R. Facklam.** 2001. *Granulicatella* and *Abiotrophia* species from human clinical specimens. J. Clin. Microbiol. **39:**3520–3523.
4. **Drancourt, M., V. Roux, P. E. Fournier, and D. Raoult.** 2004. *rpoB* gene sequence-based identification of aerobic Gram-positive cocci of the genera *Streptococcus*, *Enterococcus*, *Gemella*, *Abiotrophia*, and *Granulicatella*. J. Clin. Microbiol. **42:**497–504.
5. **Durack, D. T., E. L. Kaplan, and A. L. Bisno.** 1983. Apparent failures of endocarditis prophylaxis. Analysis of 52 cases submitted to a national registry. JAMA **250:**2318–2322.
6. **Elsayed, S., and K. Zhang.** 2004. *Gemella bergeriae* endocarditis diagnosed by sequencing of rRNA genes in heart valve tissue. J. Clin. Microbiol. **42:**4897–4900.
7. **Gephart, J. F., and J. A. Washington II.** 1982. Antimicrobial susceptibilities of nutritionally variant streptococci. J. Infect. Dis. **146:**536–539.

7a. **Glazunova, O. O., D. Raoult, and V. Roux.** 2009. Partial sequence comparison of the *rpoB*, *sodA*, *groEL* and *gyrB* genes within the genus *Streptococcus*. Int. J. Syst. Evol. Microbiol. **59:**2317–2322.

8. **Goh, S. H., D. Driedger, S. Gillett, D. E. Low, S. M. Hemmingsen, M. Amos, D. Chan, M. Lovgren, B. M. Willey, C. Shaw, and J. A. Smith.** 1998. *Streptococcus iniae*, a human and animal pathogen: specific identification by the chaperonin 60 gene identification method. J. Clin. Microbiol. **36:**2164–2166.
9. **Goh, S. H., R. R. Facklam, M. Chang, J. E. Hill, G. J. Tyrrell, E. C. Burns, D. Chan, C. He, T. Rahim, C. Shaw, and S. M. Hemmingsen.** 2000. Identification of *Enterococcus* species and phenotypically similar *Lactococcus* and *Vagococcus* species by reverse checkerboard hybridization to chaperonin 60 gene sequences. J. Clin. Microbiol. **38:**3953–3959.
10. **Goh, S. H., S. Potter, J. O. Wood, S. M. Hemmingsen, R. P. Reynolds, and A. W. Chow.** 1996. HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. J. Clin. Microbiol. **34:**818–823.
11. **Hemmingsen, S. M., C. Woolford, S. M. van der Vies, K. Tilly, D. T. Dennis, C. P. Georgopoulos, R. W. Hendrix, and R. J. Ellis.** 1988. Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature **333:**330–334.
12. **Hill, J. E., A. Paccagnella, K. Law, P. L. Melito, D. L. Woodward, L. Price, A. H. Leung, L. K. Ng, S. M. Hemmingsen, and S. H. Goh.** 2006. Identification of *Campylobacter* spp. and discrimination from *Helicobacter* and *Arcobacter* spp. by direct sequencing of PCR-amplified cpn60 sequences and comparison to cpnDB, a chaperonin reference sequence database. J. Med. Microbiol. **55:**393–399.
13. **Hill, J. E., S. L. Penny, K. G. Crowell, S. H. Goh, and S. M. Hemmingsen.** 2004. cpnDB: a chaperonin sequence database. Genome Res. **14:**1669–1675.
14. **Hung, W. C., J. C. Tsai, P. R. Hsueh, J. S. Chia, and L. J. Teng.** 2005. Species identification of mutans streptococci by *groESL* gene sequence. J. Med. Microbiol. **54:**857–862.
15. **Karenlampi, R. I., T. P. Tolvanen, and M. L. Hanninen.** 2004. Phylogenetic analysis and PCR-restriction fragment length polymorphism identification of *Campylobacter* species based on partial *groEL* gene sequences. J. Clin. Microbiol. **42:**5731–5738.
16. **Lai, C. C., C. H. Wu, J. T. Chen, and P. R. Hsueh.** 2008. Peritoneal dialysis-related peritonitis caused by *Gemella morbillorum* in a patient with systemic lupus erythematosus receiving steroid therapy. J. Microbiol. Immunol. Infect. **41:**272–274.
17. **La Scola, B., and D. Raoult.** 1998. Molecular identification of *Gemella* species from three patients with endocarditis. J. Clin. Microbiol. **36:**866–871.
18. **Liao, C. H., L. J. Teng, P. R. Hsueh, Y. C. Chen, L. M. Huang, S. C. Chang, and S. W. Ho.** 2004. Nutritionally variant streptococcal infections at a university hospital in Taiwan: disease emergence and high prevalence of beta-lactam and macrolide resistance. Clin. Infect. Dis. **38:**452–455.
19. **Liu, L. C., J. C. Tsai, P. R. Hsueh, and L. J. Teng.** 2006. Rapid differentiation between members of the anginosus group and *Streptococcus dysgalactiae* subsp. *equisimilis* within beta-hemolytic group C and G streptococci by PCR. J. Clin. Microbiol. **44:**1836–1838.
20. **Minana-Galbis, D., A. Urbizu-Serrano, M. Farfan, M. C. Fuste, and J. G. Loren.** 2009. Phylogenetic analysis and identification of *Aeromonas* species based on sequencing of the cpn60 universal target. Int. J. Syst. Evol. Microbiol. **59:**1976–1983.
21. **Roggenkamp, A., M. Abele-Horn, K. H. Trebesius, U. Tretter, I. B. Autenrieth, and J. Heesemann.** 1998. *Abiotrophia elegans* sp. nov., a possible pathogen in patients with culture-negative endocarditis. J. Clin. Microbiol. **36:**100–104.
22. **Ruoff, K. L.** 1991. Nutritionally variant streptococci. Clin. Microbiol. Rev. **4:**184–190.
23. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
24. **Teng, L. J., P. R. Hsueh, J. C. Tsai, P. W. Chen, J. C. Hsu, H. C. Lai, C. N. Lee, and S. W. Ho.** 2002. *groESL* sequence determination, phylogenetic analysis, and species differentiation for viridans group streptococci. J. Clin. Microbiol. **40:**3172–3178.
25. **Teng, L. J., P. R. Hsueh, Y. H. Wang, H. M. Lin, K. T. Luh, and S. W. Ho.** 2001. Determination of *Enterococcus faecalis groESL* full-length sequence and application for species identification. J. Clin. Microbiol. **39:**3326–3331.
26. **Thjöta, T., and J. Böe.** 1938. *Neisseria haemolysans*. A haemolytic species of *Neisseria* Treviscan. Acta Pathol. Microbiol. Scand. Suppl. **37:**527.
27. **Tsai, J. C., P. R. Hsueh, H. M. Lin, H. J. Chang, S. W. Ho, and L. J. Teng.** 2005. Identification of clinically relevant *Enterococcus* species by direct sequencing of *groES* and spacer region. J. Clin. Microbiol. **43:**235–241.
28. **Tuohy, M. J., G. W. Procop, and J. A. Washington.** 2000. Antimicrobial susceptibility of *Abiotrophia adiacens* and *Abiotrophia defectiva*. Diagn. Microbiol. Infect. Dis. **38:**189–191.
29. **Woo, P. C., A. M. Fung, S. K. Lau, B. Y. Chan, S. K. Chiu, J. L. Teng, T. L. Que, R. W. Yung, and K. Y. Yuen.** 2003. *Granulicatella adiacens* and *Abiotrophia defectiva* bacteraemia characterized by 16S rRNA gene sequencing. J. Med. Microbiol. **52:**137–140.
30. **Woo, P. C., S. K. Lau, A. M. Fung, S. K. Chiu, R. W. Yung, and K. Y. Yuen.** 2003. *Gemella* bacteraemia characterised by 16S ribosomal RNA gene sequencing. J. Clin. Pathol. **56:**690–693.
31. **Woo, P. C., K. H. Ng, S. K. Lau, K. T. Yip, A. M. Fung, K. W. Leung, D. M. Tam, T. L. Que, and K. Y. Yuen.** 2003. Usefulness of the MicroSeq 500 16S ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. J. Clin. Microbiol. **41:**1996–2001.