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Horizontal Transfer of Segments of the 16S rRNA Genes between Species of the *Streptococcus anginosus* Group

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The nature in variation of the 16S rRNA gene of members of the *Streptococcus anginosus* group was investigated by hybridization and DNA sequencing. A collection of 708 strains was analyzed by reverse line blot hybridization. This revealed the presence of distinct reaction patterns representing 11 different hybridization groups. The 16S rRNA genes of two strains of each hybridization group were sequenced to near-completion, and the sequence data confirmed the reverse line blot hybridization results. Closer inspection of the sequences revealed mosaic-like structures, strongly suggesting horizontal transfer of segments of the 16S rRNA gene between different species belonging to the *Streptococcus anginosus* group. Southern blot hybridization further showed that within a single strain all copies of the 16S rRNA gene had the same composition, indicating that the apparent mosaic structures were not PCR-induced artifacts. These findings indicate that the highly conserved rRNA genes are also subject to recombination and that these events may be fixed in the population. Such recombination may lead to the construction of incorrect phylogenetic trees based on the 16S rRNA genes.

Based on 16S rRNA gene sequence analysis, viridans streptococci are now separated into six genogroups: the pyogenic, mitis, bovis, salivarius, mutans, and anginosus groups (13). The anginosus group, also designated the *Streptococcus anginosus* group (SAG), is separated into three distinct species: *Streptococcus anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* (22). Several studies have shown that these species are important human pathogens with the capacity to cause purulent infections and abscesses (3). The three species belonging to the anginosus group are notoriously difficult to identify due to their heterogeneous biochemical and serological characteristics. Furthermore, the clinical spectrum associated with infection with members of the anginosus group also varies considerably. For this reason some scientists have resorted to genotypic rather than phenotypic characterization to differentiate these species (4, 8, 10). DNA-DNA reassociation data showed that the SAG indeed can be separated into three taxonomically distinct species but that there is considerable heterogeneity within each species (11, 22). Analysis of the 16S rRNA gene has been shown to be of value in identifying bacterial species, particularly for fastidious and uncultivable bacteria. Several research groups have used DNA sequencing of the 16S rRNA gene and hybridization with species-specific DNA probes to identify the species of the anginosus group. In previous studies we showed that among the species of the SAG, heterogeneity exists even within the well-conserved 16S rRNA gene (9, 10). In this study we show that the 16S rRNA genes of the members of the SAG show mosaic-like structures. These structures suggest that the various species within the SAG may exchange DNA fragments, including parts of the taxonomically important 16S rRNA gene.

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MATERIALS AND METHODS

Bacterial strains. In this study the following type strains were used: *S. anginosus* ATCC 33397^T (= NCTC 10713^T), *S. constellatus* ATCC 27823^T (= NCTC 11325^T = NCDO 2226^T), and *S. intermedius* ATCC 27335^T (= NCTC 11324^T = NCDO 2227^T). The other 705 streptococcal strains used for this study were unique strains that were recovered from clinical specimens submitted for culture at the microbiological laboratory of the University Hospital of Maastricht. They were identified as belonging to the SAG if they produced acetoin in the Voges-Proskauer test, hydrolyzed arginine, and failed to utilize sorbitol. The strains were stored at −70°C on porous beads in cryopreservative (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). Before inclusion in the study, they were retrieved, checked for purity, and subcultured to allow recovery from freezer storage. Hemolysis was assessed by observation of the subsurface growth in stabbed sheep blood agar plates. Both α- and γ-hemolysis were recorded as nonhemolytic. Strains were allocated to one of the following broad anatomic categories: head and neck region (including the oral cavity), thoracic cavity (including the respiratory tract), abdomen, genital tract, urinary tract, skin, bone and soft tissues, and the blood. Strains recovered from neonatal surveillance cultures were separately recorded but grouped together with the strains from the genital tract. Strains were considered infection related based on the clinical data retrieved from the laboratory request form and the hospital information system and on the laboratory data, such as the nature of the sample and the predominance of leukocytes on the Gram stain. If clinical evidence was not conclusive or if the specimen might have been contaminated by saprophytic flora, the SAG strain was considered colonizing flora.

Preparation of lysates for PCR-based analysis. For characterization on the 16S rRNA level, strains were plated on blood agar base supplemented with 5% (vol/vol) sheep blood, checked for purity, and streaked on secondary plates. The cells from the secondary plates were transferred into a microcentrifuge tube and washed twice, first in a 100-μl volume and subsequently in a 900-μl volume of 10 mM Tris–150 mM NaCl buffer (pH 8.0). The supernatant was discarded, and the cells were resuspended in 100 μl of TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8.0]) and heated at 99°C for 10 min to lyse the cells and release total DNA.

PCR amplification of the 16S rRNA gene. Primers 16S8FE and B-16S1523RB were used to amplify the virtually complete 16S rRNA gene (10). Amplification was done in 25-μl reaction volumes, in an OmniGene Thermal Cycler (Hybaid Omnigene; Hybaid Ltd., Middlesex, United Kingdom). Each sample contained 10 pmol of both primers, 0.25 U of super *Taq* DNA polymerase (HT Biotechnology, Cambridge, United Kingdom), and standard amounts of amplification reagents (200 mM [each] deoxynucleoside triphosphate, 50 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100). A 25-μl overlay of sterile mineral oil was added to the tubes. The PCR program used was 3 min at 94°C, followed by 25 cycles of amplification that consisted of 20 s at 94°C, 1 min at 55°C, and 1 min at 72°C and by a final cycle of 7 min at 72°C.

TABLE 1. Oligonucleotide primers and probes used in PCR and hybridization assays

Oligonucleotide name	Oligonucleotide sequence	Target organism, GenBank accession no.	Nucleotide position	Reference
Primers				
16S8FE	GGAATTCAGAGTTTGATCMTGGYTCAG	Eubacteria	8–27	9
B-16S1523RB	5′biotin-CGGGATCCAAGGAGGTGATCCA BCCYCA	Eubacteria	1536–1555	9
Probes				
A-Ang210	5′amino-TGCAATTGCATCGCTAGTAG	*S. anginosus* ATCC33397, AF104678	222–241	10
A-Ang280	5′amino-GTAATGGCCTACCTAGG	*S. anginosus* ATCC33397, AF104678	274–290	10
A-Ang1010	5′amino-GGAAGTTTCTTCGGAACAT	*S. anginosus* ATCC33397, AF104678	1035–1053	This study
B-Ang1010	5′biotin-GGAAGTTTCTTCGGAACAT	*S. anginosus* ATCC33397, AF104678	1035–1053	This study
A-Mot1030	5′amino-TCGGGGCACTGGTGA	*S. anginosus* Motile strain 920, AF145246	1045–1059	This study
B-Mot1030	5′biotin-TCGGGGCACTGGTGA	*S. anginosus* Motile strain 920, AF145246	1045–1059	This study
A-Con210	5′amino-GTGCAAGAGCATCACTAC	*S. constellatus* ATCC27823, AF104676	221–238	9
A-Con280	5′amino-GCTCACCAAGGCAACG	*S. constellatus* ATCC27823, AF104676	280–295	9
A-Con1035	5′amino-GGCAGAGGTGACAGG	*S. constellatus* ATCC27823, AF104676	1049–1063	This study
B-Con1035	5′biotin-GGCAGAGGTGACAGG	*S. constellatus* ATCC27823, AF104676	1049–1063	This study
A-Int210	5′amino-GTGCAAATGCATCACTAC	*S. intermedius* ATCC27335, AF104671	221–238	9
A-Int280	5′amino-GCTCACCTAGGCGAC	*S. intermedius* ATCC27335, AF104671	280–294	9
A-Int1010	5′amino-GAGCTTTACTTCGGTACAT	*S. intermedius* ATCC27335, AF104671	1035–1053	This study
B-Int1010	5′biotin-GAGCTTTACTTCGGTACAT	*S. intermedius* ATCC27335, AF104671	1035–1053	This study
A-CI210	5′amino-GTGCAAAAGCATCACTAC	*S. constellatus* CI strain, AY277937	221–238	This study
B-16S8F	5′biotin-AGAGTTTGATCMTGGYTCAG	Eubacteria	8–27	This study
B-16S1523R	5′biotin-AAGGAGGTGATCCABCCYCA	Eubacteria	1536–1555	This study

Reverse line blot hybridization. The PCR-amplified 16S rRNA genes were hybridized with 5′-amino-linked oligonucleotide probes (Isogen Bioscience, Maarssen, The Netherlands). These probes directed to species-specific regions of the 16S rRNA sequences of the various members of the SAG are displayed in Table 1. Hybridization reactions were performed in a reverse line blot assay as described before (10, 12). In brief, oligonucleotide probes were covalently linked in parallel lines to an activated Biodyne C membrane (Pall Filtron, Breda, The Netherlands) by the 5′-aminogroup link. The biotin-labeled PCR products were hybridized in lines perpendicular to the oligonucleotide probe lines. After hybridization, the membranes were washed and incubated with streptavidin-peroxidase. After a final wash, hybridization on the intersection of the probe and PCR product lines was visualized by incubation with an ECL substrate (Amersham, International plc, Den Bosch, The Netherlands) and exposure of an X-ray film. After each hybridization, the PCR products were stripped from the membrane and the membrane was stored moist at 4°C until reuse. These membranes could be reused at least 10 times.

DNA isolation and Southern blotting. For isolation of genomic DNA, strains were cultured overnight in 10 ml of Todd Hewitt broth at 35°C and 5% CO_2. Cells were spun down for 5 min at 13,000 × *g* and resuspended in 100 μl of TE buffer. DNA was isolated according to the method of Willems et al. with minor modifications (23). Briefly, the suspension was incubated for 15 min at 37°C with 500 μg of lysozyme/ml and 50 U of mutanolysin (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) to weaken the cell wall. Cells were lysed by addition of 0.5% sodium dodecyl sulfate [SDS] and 100 μg of proteinase K (Roche Molecular Biochemicals, Mannheim, Germany)/ml followed by a 30-min 65°C incubation. DNA was further purified by cetyltrimethyl ammonium bromide treatment according to the protocol, and the DNA was finally resuspended in 100 μl of TE with 100 μg of RNase/ml and stored at 4°C. DNA concentrations were determined using Picogreen (Molecular Probes Europe BV, Leiden, The Netherlands).

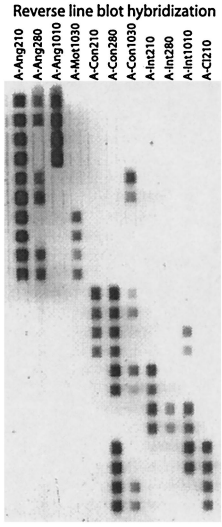
For Southern blotting, 1 to 2 μg of DNA was digested with *Hin*dIII, separated on 0.7% agarose gels, and blotted onto Hybond N+ membranes (Amersham). Hybridization with 5′-biotin-labeled oligonucleotides was performed in Dig Easy Hyb buffer (Roche Molecular Biochemicals) for 1 h at 45°C using 500 pmol of oligonucleotide probe. The membrane was washed twice for 5 min at room temperature in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate [pH 7.0])–0.1% SDS and twice for 15 min at 50°C in 0.1× SSC–0.1% SDS. The membrane was then incubated for 30 min at 42°C with peroxidase-labeled streptavidin (Roche Molecular Biochemicals) in 2× SSC–0.5% SDS followed by two 10-min washes at 42°C in 2× SSC–0.5% SDS and a 1-min wash in 2× SSC at room temperature. After the last post-streptavidine wash, hybridization was visualized using the ECL detection system described above. To obtain clear signals, exposure of the film after hybridization typically required 30 to 60 min. After development of the film, the membrane was stripped by incubating the membrane twice for 20 min at 37°C in 0.2 M NaOH–0.1% SDS. After three washes of 2 min in 2× SCC, the membrane was sealed in plastic and stored moist at 4°C until reuse.

Sequencing of the 16S rRNA gene. For DNA sequencing reactions, fluorescence-labeled-dideoxynucleotide technology was used (Applied Biosystems, Foster City, Calif.). PCR products were purified using Qiaquick PCR purification kits (Qiagen, Hilden, Germany). Sequence reactions were analyzed on ABI 377 and ABI 3700 automated DNA sequencers (Applied Biosystems). Sequencing was performed using various 16S rRNA-specific primers. The collected sequences were assembled and edited with the Seqman module of the DNAStar package (DNAStar Inc., Madison, Wis.). Multiple alignment and clustering were performed using the Kodon 1.0 software (Applied Maths, Kortrijk, Belgium).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences of the strains analyzed in this study are available in the GenBank database under accession numbers AY277937, AY277938, AY277939, AY277940, AY277941, and AY277942.

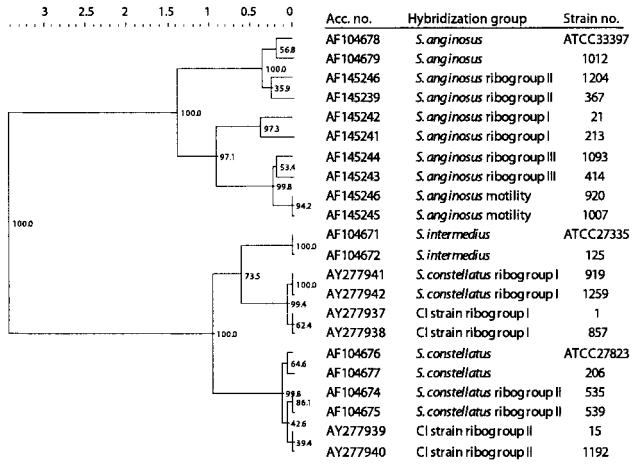
RESULTS

Patterns of reverse line blot hybridization. In previous studies on the 16S rRNA gene of SAG strains, we demonstrated the presence of various ribogroups within the SAG species (8–10). These results were based on the analysis of strains using a limited set of species-specific oligonucleotide probes. In the present study we used a collection of 708 SAG strains and hybridized the PCR products from all strains with a large set of oligonucleotide probes. These probes were based on the 16S rRNA gene sequences of the three ATCC strains (AF104678, AF104676, and AF104671), one motility strain (AF145246), and a so-called CI strain (AY277937). This resulted in the identification of 11 different hybridization groups (Fig. 1). Many of the strains yielded the expected hybridization patterns with the probes that were based on the five selected type strains. However, a large number of strains reacted with only one or two of the probes designed for identification of the particular species. In those cases the PCR product mostly reacted with one or more probes designed for identification of



SMG hybridization group	Strain no.	No. of strains with ribogroup
S. anginosus	ATCC33397	98
S. anginosus	1012	
S. anginosus ribogroup II	1204	66
S. anginosus ribogroup II	367	
S. anginosus ribogroup I	21	25
S. anginosus ribogroup I	213	
S. anginosus ribogroup III	1093	31
S. anginosus ribogroup III	414	
S. anginosus motility	1007	107
S. anginosus motility	920	
S. constellatus	ATCC27823	87
S. constellatus	206	
S. constellatus ribogroup I	1259	33
S. constellatus ribogroup I	919	
S. constellatus ribogroup II	535	73
S. constellatus ribogroup II	539	
S. intermedius	ATCC27335	92
S. intermedius	125	
CI strain ribogroup I	1	34
CI strain ribogroup I	857	
CI strain ribogroup II	15	60
CI strain ribogroup II	1192	
Total		708

FIG. 1. Identification of 11 different SAG hybridization groups by reverse line blot hybridization. The hybridization patterns in the left part of the figure were obtained by hybridizing biotin-labeled 16S rRNA gene PCR fragments in horizontal lines perpendicular to vertical lines with immobilized oligonucleotide probes. The names of the oligonucleotide probes are indicated above the vertical lines. The SAG hybridization group, the strain number used for the hybridization, and the number of strains with a particular hybridization group found among the collection of 708 strains are shown. Two representatives of each hybridization group were used in the reverse line blot.



Acc. no.	Hybridization group	Strain no.
AF104678	*S. anginosus*	ATCC33397
AF104679	*S. anginosus*	1012
AF145246	*S. anginosus* ribogroup II	1204
AF145239	*S. anginosus* ribogroup II	367
AF145242	*S. anginosus* ribogroup I	21
AF145241	*S. anginosus* ribogroup I	213
AF145244	*S. anginosus* ribogroup III	1093
AF145243	*S. anginosus* ribogroup III	414
AF145246	*S. anginosus* motility	920
AF145245	*S. anginosus* motility	1007
AF104671	*S. intermedius*	ATCC27335
AF104672	*S. intermedius*	125
AY277941	*S. constellatus* ribogroup I	919
AY277942	*S. constellatus* ribogroup I	1259
AY277937	CI strain ribogroup I	1
AY277938	CI strain ribogroup I	857
AF104676	*S. constellatus*	ATCC27823
AF104677	*S. constellatus*	206
AF104674	*S. constellatus* ribogroup II	535
AF104675	*S. constellatus* ribogroup II	539
AY277939	CI strain ribogroup II	15
AY277940	CI strain ribogroup II	1192

FIG. 2. Phylogenetic tree based on bp 8 to 1555 of the 16S rRNA gene sequences of the 11 different SAG hybridization groups. The small numbers in the dendrogram display the bootstrap values after 1,000 bootstraps. The text displays the GenBank accession number, the hybridization group, and the strain number used for sequence comparison. The genetic distance is indicated by the ruler above the dendrogram.

another species. For example, *S. anginosus* strains belonging to ribogroup II reacted with two of the three *S. anginosus* probes only and with a probe specific for *S. constellatus*. Similarly, aberrant hybridization patterns were found with many other strains, leading to the identification of 11 groups with distinct hybridization patterns among the 708 strains tested.

DNA sequence analysis of the 16S rRNA gene. To determine whether the observed hybridization patterns were caused by sequence differences or by cross-hybridization of the oligonucleotide probes, virtually the complete 16S rRNA genes of two representatives of each hybridization group were sequenced. The dendrogram in Fig. 2 shows the phylogenetic relationship of the 11 hybridization groups based on the 16S rRNA gene sequences. Due to minor differences in the 16S rRNA gene sequence, not all of the pairs of strains were clustered as identical. However, the results of the phylogenetic clustering based on the 16S rRNA gene sequence confirmed the hybridization grouping. This indicated that the unexpected hybridization results were not experimental artifacts but represented true variation in the 16S rRNA gene sequence. The bootstrapped dendrogram showed that the 11 groups were part of larger groups. There was a dichotomy of the dendrogram with a branch containing strains belonging to an *S. anginosus* group and a branch with *S. constellatus* and *S. intermedius* strains. The *S. anginosus* group could be further divided into three groups. The *S. constellatus* group was composed of two major groups, one with strains resembling the *S. constellatus* ATCC strain and the other with strains that were more closely related to the *S. intermedius* ATCC strain.

The differences in the 16S rRNA gene sequences were restricted to a number of domains, three of which were covered by the oligonucleotide probes used in this study. The multiple alignment of the three domains of the 22 sequences used for the phylogenetic tree are shown in Fig. 3. The first domain covers the region where CI strains could be distinguished from *S. constellatus* and *S. intermedius* strains based on single base changes. The second domain allowed the distinction between *S. constellatus* and *S. intermedius* and displayed minor sequence variation in the *S. anginosus* group. This sequence variation led to the loss of the hybridization signal with the A-Ang280 probe in the *S. anginosus* ribogroup II and III strains. The third domain contains the region of the 16S rRNA gene where the largest degree of sequence variation was observed. In contrast to the other domains, variation in this region seemed to be caused by exchange of DNA segments between strains rather than by mutation. As an example, the *S. anginosus* ribogroup I strains carried the same sequence in this region of the 16S rRNA gene as the *S. constellatus* ATCC strain.

Southern blot hybridization. Both the reverse line blot hybridization and the DNA sequence analysis were based on PCR-derived DNA fragments. Since SAG strains carry several copies of the 16S rRNA gene and one or more of these copies may have different DNA sequences, PCR might result in chimeric structures, leading to the unexpected hybridization and sequencing results. To exclude this possibility, Southern blots were made using *Hin*dIII-digested genomic DNA from representatives of 10 of the 11 hybridization groups. We were unable to digest CI Ribogroup II strains with *Hin*dIII and had to use of *Bam*HI for Southern blot analysis of this particular strain. The genomic blot was hybridized with a biotin-labeled species-specific oligonucleotide probe, and after development of the film the probe was stripped off and the blot was hybridized with the next probe. In this manner, the blot was hybridized with oligonucleotide probes based on the 5′ and 3′ ends of the 16S rRNA gene and with probes specific for *S. anginosus*, *S. anginosus* motility, *S. constellatus*, and *S. intermedius*. The species-specific probes were based on the third variable domain of the

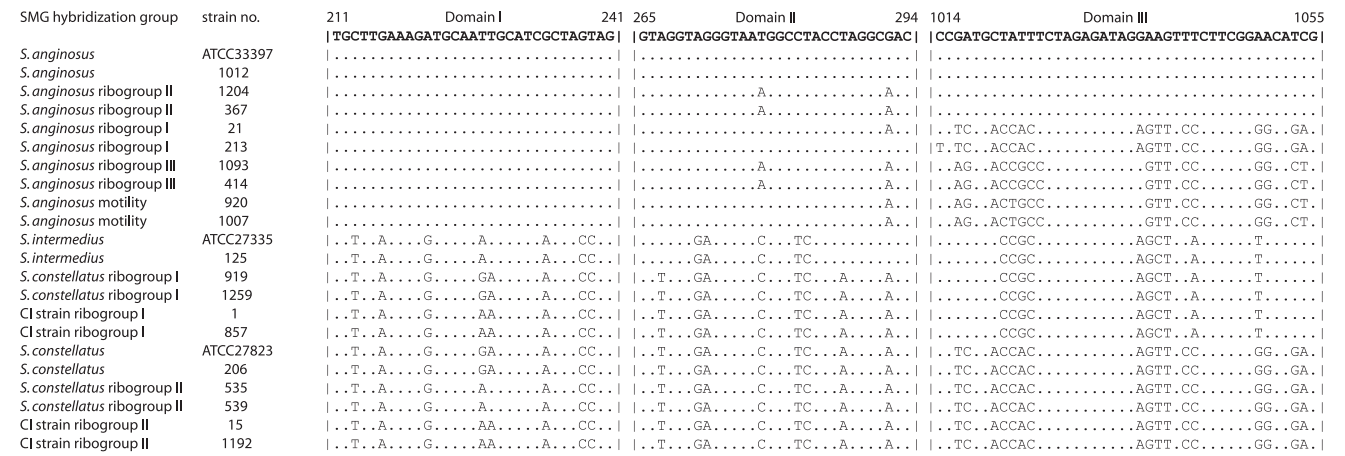


FIG. 3. Multiple alignment of three variable domains of the 16S rRNA gene sequences. The top (bold) sequence is the *S. anginosus* (ATCC 33397) sequence and was used as a consensus. The numbers at the top denote the coordinates of the 16S rRNA gene where the three domains are located.

16S rRNA gene. The results of the consecutive hybridizations are displayed in Fig. 4. The hybridization results showed that all SAG strains carried two to four copies of the 16S rRNA gene. Within a single strain, each copy of the 16S rRNA gene carried the same DNA sequence in the highly variable third domain. Therefore, it can be concluded that the unexpected hybridization and sequence results were not caused by experimental artifacts but represented true sequence changes in the 16S rRNA genes.

The genomic blot carried two strains (lanes 5 and 10) with identical restriction fragment length polymorphism patterns, often referred to as ribotypes. However, these lanes contained genomic digests from two different species with considerable differences in the 16S rRNA genes, CI strain ribogroup I (strain 28) and *S. constellatus* ribogroup II (strain 539), respectively. This is a remarkable result, since ribotyping is often used to differentiate strains within a single species.

Other characteristics of the strains. The phenotypical and clinical characteristics of the *S. anginosus*-related ribogroups have been described previously (10). In addition, the *S. constellatus* ribogroup II strains have been described before (9, 11). The latter strains represented hemolytic SAG isolates that phenotypically presented as *S. intermedius* but did not lyse human red blood cells. Among the SAG species or variants, *S. intermedius* was the most homogeneous at the 16S rRNA gene level. In contrast, the *S. constellatus* group contained several hybridization groups, namely CI strains ribogroup I, CI strains ribogroup II, and *S. constellatus* ribogroup I. All three groups shared the anatomic distribution and clinical significance of the *S. constellatus* species as well as their hemolytic characteristics (Table 2).

DISCUSSION

In this study we have shown that the 16S rRNA genes of members of the SAG display an unusual sequence diversity that is most likely caused by lateral transfer and recombination. We have found at least 11 different hybridization groups by reverse line blot hybridization using oligonucleotide probes targeted at three different regions in the 16S rRNA gene. DNA sequence analysis of the complete 16S rRNA gene of representatives of these hybridization groups confirmed this varia-

TABLE 2. Hemolytic and clinical characteristics of SAG strains used in the study

Strain or group[a]	% of strains											
	Hemolysis		Clinical significance			Site of sampling						
	−	+	Abscess	Infection	Colonizing flora	Abdomen	Thorax	Genital region	Head and neck	Skin and soft tissue	Blood	Urinary tract
S. anginosus	90	10	53	26	21	39	9	3	7	11	20	10
S. anginosus RG II	62	38	36	23	41	44	21	8	10	2	11	5
S. anginosus RG I	96	4	54	42	4	78	0	0	0	4	13	4
S. anginosus RG III	96	4	61	26	13	35	0	6	10	19	16	13
S. anginosus motility	97	3	10	24	66	11	1	36	1	5	1	46
S. intermedius	95	5	68	26	6	25	15	2	20	18	20	0
S. constellatus RG I	68	32	42	21	38	21	25	8	8	17	21	0
CI strain RG I	42	58	33	30	36	30	21	9	12	15	3	9
S. constellatus	21	79	33	26	42	26	33	2	13	9	15	2
S. constellatus RG II	2	98	24	16	60	19	36	3	19	7	6	11
CI strain RG II	13	87	38	14	48	25	38	9	13	7	2	5

[a] RG, ribogroup.

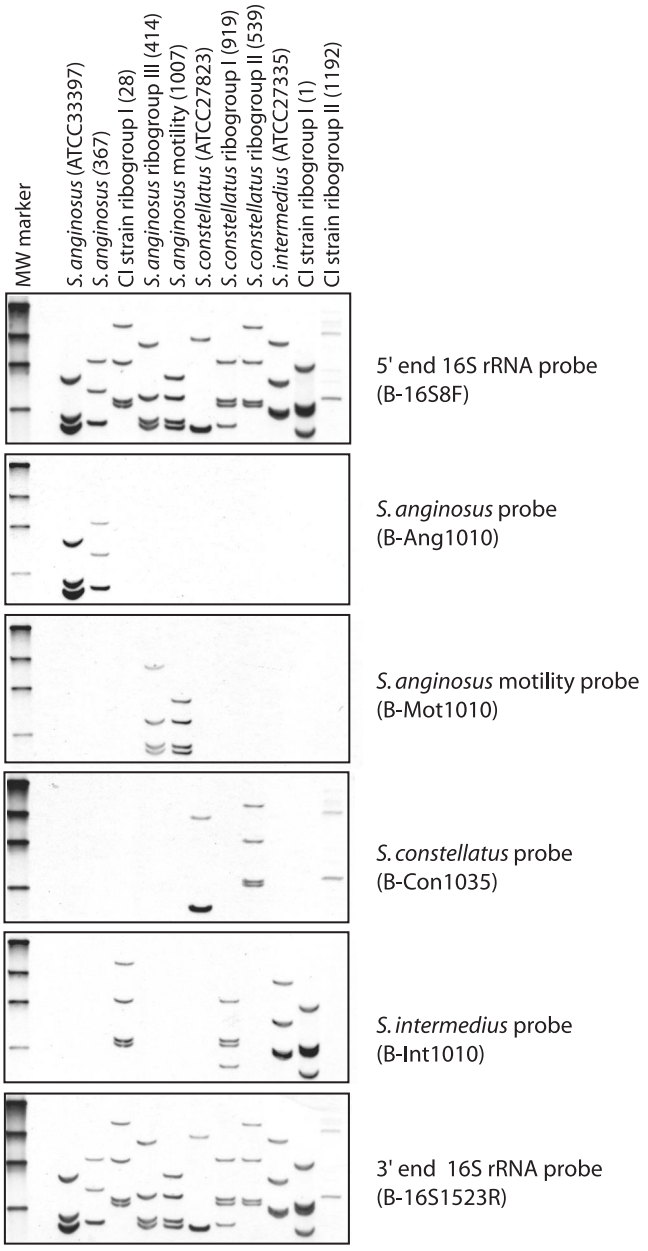


FIG. 4. Southern blot hybridizations of a number of representative strains of the various hybridization groups with species-specific probes. Genomic DNA from the SAG strains was digested with *Hin*dIII with the exception of the CI strain ribogroup II (strain 1192), which was digested with *Bam*HI. The membrane with the fragments was hybridized with a biotin-labeled oligonucleotide probe. After analysis the probe was stripped off, and subsequently the membrane was hybridized with the next oligonucleotide probe until all six probes were used. The blot contains a biotin-labeled molecular weight marker of which the 23.1-, 9.4-, 6.6-, and 4.4-kb bands are visible in the image.

tion and showed that the region from bp 1014 to 1055 displayed the highest degree of variation. DNA segments from this region seem to have been exchanged between the various species belonging to the SAG.

It is generally believed that lateral transfer and recombination do not lead to rapid changes in rRNA genes. One of the main reasons would be that the tertiary structure of the ribosomal RNAs is essential for the association with components of the translation apparatus, such as the ribosomal proteins. Changes in primary sequence of the rRNA gene may alter the folding of the molecule, and such changes may inhibit association with the ribosomal proteins. During evolution, gradual changes may have been introduced in the rRNA genes and ribosomal protein genes, leading to the slow emergence of new ribosomal complexes. This hypothesis is supported by the fact that mutations in stems of the stem-loop structures in the 16S rRNA gene often are associated with mutations in opposing parts of the rRNA strand, leading to restoration of the stem structure. Bacterial phylogenies are often based on the dogma that the rRNA genes undergo only a very gradual change. The rRNA genes are universally present in all organisms, can be easily obtained using PCR with universal primers, and are easy to sequence. For this reason, the use of, particularly, the 16S rRNA gene sequences for phylogeny and identification studies has become extremely popular and has led to the reconstruction of the tree of life (24, 25).

The results of our experiments clearly show that at least some bacterial species may have a history of lateral transfer and recombination of segments of the 16S rRNA genes. The rapid variation in the 16S rRNA genes may result in incorrect phylogenetic trees that are derived from these ribosomal gene sequences. Furthermore, it may lead to misidentification if oligonucleotide probes based on the 16S rRNA genes are used for species identification. This is not the first report on evidence for lateral transfer of segments of ribosomal rRNA genes. Analysis of rRNA genes suggests that lateral transfer of ribosomal gene segments also occurs in *Rhizobium*, *Aeromonas*, *Bradyrhizobium*, and actinomycetes (2, 16, 20, 21). There also have been reports on transfer of complete rRNA operons, both within and between species (14, 26). Our data and data from the cited studies show that bacteria can be subjected to significant changes in their rRNA genes without apparent loss of fitness and virulence. In a recently published study, researchers deleted all of the seven chromosomal rRNA operons of *Escherichia coli* (1). In the experiment, they introduced plasmids carrying the rRNA operons of either *Salmonella enterica* serovar Typhimurium or *Proteus vulgaris* to provide the *E. coli* with rRNA genes, and this yielded viable and apparently fit *E. coli*. This result indicates that a large degree of variation of the 16S rRNA gene is allowed for without affecting fitness.

When segments of the 16S rRNA gene are transferred from one species to another, this is likely to happen in just one of the copies of the ribosomal operons first. This would lead to strains with two different types of 16S rRNA genes, as has been found for a *Mycobacterium terrae* strain (15). However, by Southern blot hybridization we have shown that all copies of the 16S rRNA gene in the individual SAG strains that may have undergone lateral transfer of 16S rRNA gene segments carried the same signature sequence. This indicates that homogenization of the 16S rRNA genes must have taken place within the strains after the lateral transfer. A recent study showed that at least for *E. coli*, homogenization of 16S rRNA genes can take place quite rapidly (5).

The variation of the 16S rRNA genes that we detected further complicates the species identification of the members of the SAG. It suggests that species identification based solely on the 16S rRNA gene sequences may lead to misidentifica-

tions. This is particularly true when only short segments of the gene are used, e.g., in hybridization assays using oligonucleotide probes. Nevertheless, detection and identification based on this 16S rRNA gene have proven to be extremely helpful, particularly for the identification of fastidious bacteria and for bacteria that cannot be grown in vitro (18). Is lateral transfer of 16S rRNA gene segments a rare event? This is uncertain, and to our knowledge only a few papers have been published on this subject (2, 16, 20, 21). In recent studies, interstrain variation in the 16S rRNA gene of *Neisseria meningitidis* was used as a molecular typing tool (17, 19). In these studies, 16S typing was the most sensitive and specific typing tool to discriminate outbreak-related isolates from sporadic isolates. The performance was even better than that of multilocus sequence typing. Analysis of the *N. meningitidis* 16S sequence data also showed that the 16S rRNA gene may recombine as frequently as the housekeeping genes but that the pool of functional 16S rRNA sequences is limited. Apparently, most recombinants are not fixed in the population due to their negative effect on fitness. Like *N. meningitidis*, some SAG strains are naturally competent and can take up DNA from the environment (6, 7). Furthermore, different SAG strains may simultaneously be part of the colonizing or infecting flora, thus creating the conditions required for horizontal transfer of genes or gene segments.

In the present study, we have demonstrated that members of the SAG have diverse composition of the 16S rRNA gene. We were able to distinguish at least 11 different ribosomal groups. It is clear that these groups do not all represent distinct species but mainly represent variants belonging to a limited number of species. Nevertheless, some of the variants display aberrant phenotypical behavior and distinct clinical manifestations. Three large groups could be discerned. The *S. anginosus* group was comprised of strains with various 16S rRNA gene sequences and diverse phenotypical and clinical relevance. The *S. constellatus* group also was composed of several different hybridization groups but displayed limited phenotypical variation and no differences in clinical relevance. *S. intermedius* was the only homogeneous group at the 16S level, as well as on the phenotypical and clinical level. These results showed that 16S rRNA sequences do not necessarily reflect the phenotypic and clinical significance of the strains. This may be particularly true for organisms like SAG that exchange segments of the 16S rRNA gene.

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