Phylogenetic Relationship of *Gemella morbillorum* to *Gemella haemolysans*

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Partial 16S rRNA gene sequences (16S rDNA) of *Gemella morbillorum* and *Gemella haemolysans* were determined by sequencing polymerase chain reaction-amplified DNA. A phylogenetic analysis of 53 eubacterial 16S rRNA sequences grouped the gemellae on a distinct branch separate from the 18 members of the genus *Streptococcus*. DNA-DNA hybridization results indicate that the two gemellae are related at the genus level but are not a single species.

Gemella haemolysans, the original member of the genus *Gemella*, was first described in 1938 as *Neisseria haemolysans* by Thjötta and Böe (38). Several studies showed that *N. haemolysans* was biochemically quite different from other *Neisseria* species (for a review, see reference 8), and Berger proposed the new genus *Gemella* (7).

Gemella morbillorum was originally isolated in 1917 by Tunnick from the blood of 42 patients during the early stages of measles (40). She later named the gram-positive organism *Diplococcus rubeolae* (41). Prevot also isolated an organism from patients with measles and named it *Diplococcus morbillorum* (33). The two *Diplococcus* species were apparently identical, and Tunnick adopted the name *D. morbillorum*. Although *D. morbillorum* could be grown under aerobic conditions after several transfers, it was primarily anaerobic and was transferred to the genus *Peptostreptococcus* by Smith (36). *Peptostreptococcus morbillorum*, however, was found to have lactic acid as its major by-product of metabolism, and Holdeman and Moore (26) transferred it to the genus *Streptococcus*.

The 16S rRNA oligonucleotide catalog of *Streptococcus morbillorum* closely resembled that of *G. haemolysans* (31), and in 1988, Kilpper-Bälz and Schleifer's DNA-DNA filter hybridization results and G+C content analysis compelled them to propose that *S. morbillorum* be transferred to the genus *Gemella* as *G. morbillorum* comb. nov. (30). The G+C content of *S. morbillorum* was 30 mol%, compared with 33 mol% for *G. haemolysans* and 38 to 46 mol% for streptococci (31).

G. morbillorum and *G. haemolysans* were found to be morphologically and physiologically very similar, and Facklam and Wilkinson concluded that the two species were actually identical (23). Later studies, however, demonstrated that *G. haemolysans* could be differentiated from *G. morbillorum* by biochemical assays including nitrite reduction (see references 8 and 9). Both species are considered commensal with humans, but each has been implicated as a causative agent of endocarditis (5, 13, 29, 32). *G. haemolysans* has been reported in clinical cases of septicemia and meningitis and an infection of a total knee arthroplasty (21, 22).

We sought to further define the phylogenetic relationship of *G. morbillorum* to *G. haemolysans* by determining and comparing the sequences of the 16S rRNA gene (16S rDNA) from the type strains of both species. The two 16S rRNA

gene sequences were then aligned with 16S rRNA (rDNA) sequences of 51 eubacterial species representing 11 genera, and a phylogenetic tree was constructed. The tree and DNA-DNA hybridization results confirm the separation of the genera *Gemella* and *Streptococcus* and show that *G. haemolysans* and *G. morbillorum* are unique species.

MATERIALS AND METHODS

Bacteria. Bacterial cultures used in this study were *G. morbillorum* ATCC 27824^T, *G. haemolysans* ATCC 10379^T, and *Aerococcus viridans* ATCC 11563^T.

Preparation of genomic DNA for PCR or hybridization. *G. morbillorum* was grown for 24 h at 37°C on Trypticase soy agar supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.). Cells were harvested by scraping with an inoculating loop and were suspended in 500 µl of 0.2 M sodium acetate. The bacteria were washed once with 0.2 M sodium acetate and suspended in 500 µl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Ten microliters of mutanolysin (Sigma Chemical Co., St. Louis, Mo.) (1 mg/ml) was added to the resuspended bacteria, and the tube was incubated at 37°C for 1 h. The suspension was then transferred to a thermal cycler and heated at 100°C for 10 min. Cellular debris was pelleted by centrifugation, and 10 µl of the resulting supernatant was used in each 100-µl polymerase chain reaction (PCR).

Chromosomal DNA for DNA-DNA hybridization was prepared from each strain by growing 1 liter of culture in Todd-Hewitt broth supplemented with yeast extract (Difco Laboratories, Detroit, Mich.) at 37°C overnight. The bacteria were washed in SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and suspended in 20 ml of SSC. An equal volume of glass beads was added to the suspension, and the bacteria were disrupted in a Braun homogenizer (B. Braun Biotech International, Allentown, Pa.) for 2 min. After the beads were allowed to settle, the supernatant was recovered, extracted once with phenol and twice with CHCl₃, and precipitated with ethanol overnight. The DNA was washed with unlabeled 70% ethanol and suspended in 900 µl of H₂O. One hundred microliters of 10× STE (1× STE is 100 mM NaCl, 10 mM Tris, and 1 mM EDTA [pH 7.4]) was added to the DNA suspension. RNase (Sigma) was added to a final concentration of 50 µg/ml, and the suspension was heated at 60°C for 1 h. Pronase (Sigma) was then added to a final concentration of 50 µg/ml, and the suspension was heated at 37°C for 1 h. The DNA was extracted as described above and resuspended in H₂O. The optical density at 260 nm of the

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DNA suspension was determined, and the concentration of the DNA was adjusted to 1 mg/ml.

Template preparation and sequencing. Primers fd1 (5'-agagtttgatcctggctcag) and rD1 (5'-aaggagtgatccagcc) (44) were used to amplify the 16S rDNA as previously described (19). Each 100- μ l reaction mixture contained 10 μ l of supernatant from the *G. morbillorum* bacterial cell lysate or 10 μ l of *G. haemolysans* DNA diluted to 1 μ g/ml. Ten reaction mixtures were pooled, and 10 μ l of the pool was run on a 1% agarose gel to check concentration and purity. Purification of the PCR product with streptavidin-coated magnetic beads (Dynabeads M-280; Dynal, Inc. Great Neck, N.Y.) has been described elsewhere (19).

Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio) was used to sequence each strand of the PCR product by the dideoxy chain termination method described by Sanger et al. (35). 35 S-dATP was purchased from New England Nuclear (Dupont Corporation, Wilmington, Del.). The standard protocols were followed with 7 μ l of beads or supernatant containing template DNA in each reaction. Ten of the primers used for sequencing were derived from Stackebrandt and Charfreitag (37). Primer 235 (5'-GCACCG CAAAGCCATCTC) was derived from the *G. haemolysans* 16S rDNA sequence as it became known (the present study).

Phylogenetic analysis. Partial 16S rDNA consensus sequences for *G. haemolysans* and *G. morbillorum* were determined after results from three or more Sequenase reactions from each strand of DNA template were aligned and edited by using the DNASTAR (Madison, Wis.) software package. The two sequences, representing approximately 98% of their respective genes, were then aligned with 51 other eubacterial 16S rDNA sequences or 16S rRNA sequences representing 11 genera by the Pileup program in the Genetics Computer Group sequence analysis package (20). The accession numbers, strain designations, and references for the sequences used in the alignment are listed in Table 1. The alignment was edited by removing approximately 150 nucleotides at the 5' end of the sequences including the V1 region (nomenclature of Gray et al. [25]) and by removing the 3' terminus including the V5 region. The resulting length of 1,350 nucleotides corresponded to bases 149 through 1477 in the *Escherichia coli* nomenclature described by Brosius et al. (12). Phylogenetic analyses were based on a distance matrix output generated by the PHYLIP version 3.4 DNADIST program (24) using the nucleotide substitution model described by Jukes and Cantor (28). The neighbor-joining method described by Saitou and Nei (34) was used to generate unrooted trees assuming no evolutionary clock. The distance matrix input was taken in random order. The outgroup for this analysis was *Anacystis nidulans* 16S rDNA (39). A rooted phenogram was then constructed (Drawgram; PHYLIP version 3.4).

DNA-DNA hybridization. Sonicated genomic DNAs from *G. morbillorum*, *G. haemolysans*, and *A. viridans* were used in DNA-DNA hybridization assays in a modification of the methods described by Brenner et al. (11). One microgram of *G. haemolysans* DNA was labeled with [35 P]dCTP in a nick translation system (GIBCO-BRL, Gaithersburg, Md.). Unincorporated label was removed by passing the reaction through a nick column (Sephadex G-50; Pharmacia, Piscataway, N.J.). Labeled *G. haemolysans* DNA was then diluted to 0.33 μ g/ml (specific activity was 4×10^5 to 2.4×10^6 cpm/ μ g) in 0.28 M phosphate buffer containing 150 μ g of unlabeled homologous or heterologous DNA per ml. A negative control tube contained only labeled *G. haemolysans* DNA in 0.28 M phosphate buffer. Hybridization

TABLE 1. Sources and accession numbers of 16S rRNA (rDNA) sequences used in this study

Organism	Strain	Accession no.	Reference or source ^a
<i>Aerococcus urinae</i>	NCFB 2893 ^T	M77819	2
<i>Aerococcus viridans</i>	ATCC 11563 ^T	M58797	NP
<i>Alloiococcus otitis</i>	NCFB 2890	X59765	1
<i>Anacystis nidulans</i>		X03538	39
<i>Bacillus anthracis</i>	Sterne	X55059	3
<i>Bacillus cereus</i>	NCDO 1771 ^T	X55060	3
<i>Bacillus subtilis</i>	NCDO 1769	X60646	4
<i>Brochothrix campestris</i>	ATCC 43754 ^T	X56156	17
<i>Brochothrix thermosphacta</i>	NCDO 1676 ^T	X56155	17
<i>Carnobacterium divergens</i>	NCDO 2763 ^T	X54270	42
<i>Carnobacterium gallinarum</i>	NCFB 2766 ^T	X54269	42
<i>Carnobacterium mobile</i>	NCFB 2765 ^T	X54271	42
<i>Carnobacterium piscicola</i>	NCDO 2762 ^T	X54268	42
<i>Enterococcus faecium</i>	NCDO 942 ^T		46
<i>Enterococcus hirae</i>	NCDO 1258 ^T		46
<i>Gemella haemolysans</i>		M58799	NP
<i>Gemella haemolysans</i>	ATCC 10379 ^T	L14326	TS
<i>Gemella morbillorum</i>	ATCC 27824 ^T	L14327	TS
<i>Helcococcus kunzii</i>	NCFB 2900 ^T		15
<i>Lactobacillus acidophilus</i>	NCDO 1748 ^T	X61138	16
<i>Lactobacillus delbrueckii</i>	DSM 20074 ^T	M58814	NP
<i>Lactococcus garvieae</i>	NCDO 2155 ^T	X54262	14
<i>Lactococcus piscium</i>	NCFB 2778 ^T	X53905	47
<i>Lactococcus plantarum</i>	NCDO 1869 ^T	X54259	14
<i>Lactococcus raffinolactis</i>	NCDO 617 ^T	X54261	14
<i>Lactococcus lactis</i>	NCDO 2118 ^T	X54260	14
<i>Leuconostoc mesenteroides</i>	DSM 20343 ^T	M23035	49
<i>Leuconostoc</i>	DSM 20288 ^T	M23033	49
<i>paramesenteroides</i>			
<i>Listeria innocua</i>	NCTC 11288 ^T	X56152	17
<i>Listeria monocytogenes</i>	ATCC 35152	M58822	17
<i>Pediococcus damnosus</i>	NCDO 1832 ^T		18
<i>Pediococcus parvulus</i>	NCDO 1634 ^T		18
<i>Pediococcus pentosaceus</i>	DSM 20336	M58834	NP
<i>Streptococcus acidominimus</i>	NCDO 2025 ^T	X58301	6
<i>Streptococcus alactolyticus</i>	NCDO 1091 ^T	X58319	6
<i>Streptococcus anginosus</i>	NCTC 10713 ^T	X58309	6
<i>Streptococcus bovis</i>	NCDO 597 ^T	X58317	6
<i>Streptococcus cricetus</i>	NCDO 2720 ^T	X58305	6
<i>Streptococcus equi</i>	NCDO 2493 ^T	X58314	6
<i>Streptococcus equinus</i>	NCDO 1037 ^T	X58318	6
<i>Streptococcus hyointestinalis</i>	DSM 20770 ^T	X58313	6
<i>Streptococcus iniae</i>	NCDO 2772 ^T	X58316	6
<i>Streptococcus intermedius</i>	NCTC 11324 ^T	X58311	6
<i>Streptococcus mutans</i>	NCTC 10499 ^T	X58303	6
<i>Streptococcus parasanguis</i>	ATCC 15912 ^T	X53652	45
<i>Streptococcus pneumoniae</i>	NCTC 7465 ^T	X58312	6
<i>Streptococcus pyogenes</i>	NCDO 2381 ^T	X59029	6
<i>Streptococcus rattus</i>	NCDO 2723 ^T	X58304	6
<i>Streptococcus salivarius</i>	ATCC 13419	M58839	NP
<i>Streptococcus sanguis</i>	NCTC 7863 ^T	X53653	45
<i>Streptococcus sobrinus</i>	DSM 20742 ^T	X58307	6
<i>Tetragenococcus halophilus</i>	NCBI 12011 ^T		18
<i>Vagococcus fluvialis</i>	NCDO 2497 ^T	X54258	14
<i>Vagococcus salmoninarum</i>	NCFB 2777 ^T	X54272	42

^a TS, this study; NP, submitted to GenBank as unpublished.

was carried out at 55°C (optimal) or 70°C (stringent). The 55°C reaction conditions are such that the DNA reaches 100 C₀t_s by 16 h. After renaturation, the single-stranded DNA was separated from the double-stranded DNA by passing the samples through a hydroxylapatite column. The hydroxylapatite-bound DNA was eluted by sequentially increasing the wash temperature by 5°C.

G.h.	GATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAAGTTTCTGGTGCT	70
G.m.		
G.h.	TGCACCGGAAAACTTAGCGGCGAAGCGGTGAGTAACACGTAAGAAGCTGCCTCATAGACTGGGCAAC	140
G.m.	TA.....	
G.h.	TATTGGAAACGATAGCTAATACCGGATAACAGCATTAACTGCATGGTTGATGTTTAAAGTTGGTTTTC	210
G.m.	T...TCTC...AGA...A...A...	
G.h.	TAACACTATGAGATGGCTTTGCGGTGCATTAGCTAGTTGGTGGGTAAGGCCCTACCAAGGCGACGATGC	280
G.m.	C.....	
G.h.	ATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGCGCCAGACTCCTACGGGAGGCAGC	350
G.m.		
G.h.	AGTAGGGAATCTTCCGCAATGGGCGAAAAGCCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGATTTCCGT	420
G.m.		
G.h.	TCGTAAAGCTCTGTTGTTAGGGAAGATGATTGTGTAGTAACATACACAGTAGAGACGGTACCTAACCA	490
G.m.	GA.A.....TGTA.....	
G.h.	GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATT	560
G.m.		
G.h.	GGGCGTAAAGCGCGCCAGGTGGTTTAAATAAGTCTGATGTGAAAGCCACGGCTCAACCGTGGAGGGTCA	630
G.m.		
G.h.	TTGGAACTGTTAACTTGAGTGCCAGGAGAGAAAAGTGAATTCCTAGTGTAGCGGTGAAATGCGTAGAG	700
G.m.		
G.h.	ATTAGGAGGAACACCAGTGGCGAAGCGGCTTTTGGCCTGTAACTGACACTGAGGCGCGAAGCGTGGG	770
G.m.		
G.h.	GAGCAACAGGATTAGATACCTCGGTAGTCCACGCGCTAAACGATGAGTGCTAAGTGTGGTCTCATAAG	840
G.m.		
G.h.	AGATCAGTGCTGCAGCTAACGCATTAAAGCACTCCGCGTGGGAGTACGACCGCAAGGTTGAAACTCAAAG	910
G.m.		
G.h.	GAATTGACGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAACTCGAAGCAACGCGAAGAACCTTACCA	980
G.m.		
G.h.	AGTCTTGACATACTGTGAGGACACAAGAGATTGTGTTGTTCTGACCTTTGGTTAGACACAGATACAGGTG	1050
G.m.		
G.h.	GTGCATGGTTGTCTGCTCAGCTCGTGTCTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTTATAT	1120
G.m.		
G.h.	CTAGTTGCCAGCAGTAAGATGGGACTCTAGATAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGA	1190
G.m.		
G.h.	CGTCAAATCATATGCCCTTATGACTTGGGCTACACACGTGCTACAATGGATAGGAACAAAGAGAAGCG	1260
G.m.		
G.h.	AGCTCGCGAGAGTAAGCAACCTCACAAAACCTATTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACAT	1330
G.m.	C...C...T.....	
G.h.	GAAGCTGGAATCGCTAGTAATCGCAATCAGAATGTCGCGGTGAATACGTTCCCGGGTCTTGTACACACC	1400
G.m.		
G.h.	GCCCGTCACACCACGAGAGTTTGAACACCCGAAGACGCGTGGCCTAACCTTTTAGGAGGGAGCCGGTCAC	1470
G.m.	A.....	
G.h.	GTGGGACAGATGATTGGGGTGAAGTCGTAACAAAGTAGCCGTATCGGAAGGTGCGGCTGGATCACCT	1537
G.m.		

FIG. 1. Aligned 16S rRNA sequences from *G. haemolysans* (G.h.) and *G. morbillorum* (G.m.). Consensus nucleotides are indicated by periods.

The thermal elution midpoint, the temperature at which 50% of the double-stranded DNA had melted and was eluted from the hydroxylapatite bed, was determined for the homologous and heterologous hybridization reactions. Compared with the homologous reaction, a decrease in the thermal elution midpoint of a heterologous reaction by 1°C is indicative of approximately 1% mismatched base pairs (10), i.e., 1% divergence.

DNA relatedness between *G. haemolysans* and *A. viridans* or *G. morbillorum* was determined by assaying the percentage of double-stranded DNA in the total DNA of each reaction (binding ratio), subtracting the nonassociated labeled DNA, and relating the heterologous difference to the homologous difference (relative binding ratio). The relative binding ratios for the 55 and the 70°C reaction mixtures were determined.

Nucleotide sequence accession numbers. The 16S rDNA sequences were deposited in GenBank under the accession numbers L14327 (*G. morbillorum*) and L14326 (*G. haemolysans*).

RESULTS AND DISCUSSION

PCR amplification of the 16S rRNA gene (16S rDNA) from *G. haemolysans* or *G. morbillorum* generated a single product for each organism that migrated at approximately 1,500

bp. The PCR products were sequenced, and the two sequences were aligned. The alignment (Fig. 1) showed 24 differences over the 1,536-base length, giving a similarity value of 98.44%. Twenty-three of the 24 nucleotide differences were in typically variable regions of the rRNA sequence (11). Variable region 1 (V1) contained 2 bases that were not homologous; totals of 10, 7, 3, and 1 bases were located in V2, in V6, in V9, and in V5, respectively. Both sequences exhibited all 40 of the specific nucleotides of the 16S rRNA signature characteristic of the gram-positive eubacteria with a low G+C content (48).

TABLE 2. DNA relatedness between *G. haemolysans* and *G. morbillorum* or *A. viridans*

Source of unlabeled DNA	% Relatedness to labeled DNA from <i>G. haemolysans</i> type strain ATCC 10379		% Divergence
	55°C	70°C	
<i>G. haemolysans</i> ATCC 10379 ^T	100.0	100.0	0.0
<i>G. morbillorum</i> ATCC 27824 ^T	21.5	10.5	10.7
<i>A. viridans</i> ATCC 11563 ^T	4.2	3.5	11.9

TABLE 3. Percent similarity and K_{unc} s of partial 16S rRNA sequences

Organism	% Similarity and K_{unc} ^a															
	<i>G. haemolysans</i> ATCC 10379 ^T	<i>G. morbillorum</i> ATCC 27824 ^T	<i>A. viridans</i> ATCC 11563 ^T	<i>B. subtilis</i> NCDO 1769 ^T	<i>E. faecium</i> NCDO 942 ^T	<i>E. hirae</i> NCDO 1258 ^T	<i>L. acidophilus</i> NCDO 1748 ^T	<i>L. garvieae</i> NCDO 2155 ^T	<i>L. lactis</i> NCDO 2118 ^T	<i>L. mesenteroides</i> DSM 20343 ^T	<i>P. damnosus</i> NCTC 1832 ^T	<i>S. mutans</i> NCDO 10499 ^T	<i>S. pyogenes</i> NCDO 2381 ^T	<i>T. halophilus</i> NCBI 12011 ^T	<i>V. fluvialis</i> NCDO 2497 ^T	
<i>G. haemolysans</i> ATCC 10379 ^T		0.0165	0.1492	0.1551	0.1485	0.1489	0.1778	0.1942	0.1778	0.2245	0.2056	0.1876	0.1688	0.2402	0.1685	
<i>G. morbillorum</i> ATCC 27824 ^T	98.4 ^b		0.1570	0.1596	0.1518	0.1501	0.1806	0.1935	0.1830	0.2130	0.2088	0.1898	0.1687	0.2388	0.1711	
<i>A. viridans</i> ATCC 11563 ^T	86.5	85.8		0.1376	0.0921	0.0929	0.1478	0.1624	0.1511	0.1593	0.1564	0.1578	0.1528	0.1852	0.0990	
<i>B. subtilis</i> NCDO 1769 ^T	86.0	85.6	87.4		0.0995	0.1031	0.1496	0.1994	0.1759	0.1947	0.1626	0.1687	0.1700	0.1940	0.1256	
<i>E. faecium</i> NCDO 942 ^T	86.5	86.3	90.7	91.3		0.0041	0.1157	0.1366	0.1233	0.1607	0.1216	0.1253	0.1125	0.1437	0.0720	
<i>E. hirae</i> NCDO 1258	86.5	86.4	91.3	90.4	99.6		0.1198	0.1383	0.1229	0.1628	0.1304	0.1278	0.1105	0.1422	0.0714	
<i>L. acidophilus</i> NCDO 1748 ^T	84.2	83.9	86.6	86.4	89.3	88.9		0.1834	0.1650	0.1658	0.1468	0.1649	0.1568	0.1937	0.1412	
<i>L. garvieae</i> NCDO 2155 ^T	82.9	82.9	85.4	82.5	87.5	87.4	83.7		0.0783	0.2011	0.1960	0.1294	0.1109	0.2097	0.1668	
<i>L. lactis</i> NCDO 2118 ^T	84.2	83.8	86.3	84.3	88.6	88.7	85.2	92.6		0.1989	0.1680	0.1017	0.0974	0.2094	0.1473	
<i>L. mesenteroides</i> DSM 20343 ^T	80.6	81.4	85.6	82.5	85.5	85.4	85.1	82.4	82.5		0.2014	0.1911	0.1882	0.2257	0.1751	
<i>P. damnosus</i> NCTC 1832 ^T	82.0	81.8	86.0	85.4	88.8	88.0	86.7	82.7	84.9	82.3		0.1733	0.1664	0.2166	0.1379	
<i>S. mutans</i> NCDO 10499 ^T	83.4	83.2	85.8	84.9	88.5	88.2	85.2	88.1	90.5	83.1	84.5		0.0680	0.1985	0.1466	
<i>S. pyogenes</i> NCDO 2381 ^T	84.9	84.9	86.2	84.8	89.5	89.7	85.8	89.7	90.9	83.4	85.1	93.5		0.1970	0.1370	
<i>T. halophilus</i> NCBI 12011 ^T	79.4	79.5	83.6	82.9	86.9	87.0	82.9	81.7	81.7	80.5	81.2	82.6	82.7		0.1543	
<i>V. fluvialis</i> NCDO 2497 ^T	84.9	84.7	90.7	88.4	93.1	93.2	87.1	85.0	86.6	84.4	87.4	86.7	87.5	86.0		

^a K_{unc} , evolutionary distance value (27).^b Numbers represent similarity corrected for multiple base changes by the method described by Jukes and Cantor (28). Accession numbers and references for sequences obtained from GenBank are listed in Table 1.

FIG. 2. Phylogenetic tree based on 16S rDNA sequences using the neighbor-joining method described by Saitou and Nei (34). The bar represents 5% difference in nucleotide sequence.

The *G. haemolysans* 16S rDNA sequence was also aligned with the *G. haemolysans* 16S rRNA sequence retrieved from GenBank (Table 1) that had been determined by reverse transcriptase (data not shown). We were able to identify 60 bases that were ambiguous in the rRNA sequence; we found 1 base that differed and needed to introduce a 1-base gap at position 1487 to accommodate a guanosine in the rRNA sequence that we were unable to confirm.

DNA-DNA hybridization assay results between the total genomic DNAs of *G. haemolysans* and *G. morbillorum* are shown in Table 2. According to Wayne et al., a species is defined phylogenetically by "approximately 70% or greater DNA-DNA relatedness and with 5°C or less ΔT_m " (43). At optimal conditions, *G. haemolysans* and *G. morbillorum* showed only 21.5% DNA-DNA relatedness and a ΔT_m of 10.7°C. These results indicate that *G. haemolysans* and *G. morbillorum* are related at the genus level but not at the species level.

The sequences of the 16S rRNA genes from *G. haemolysans* and *G. morbillorum* were aligned with 51 other eubacterial 16S rRNA (rDNA) sequences retrieved from EMBL or GenBank. Before analysis of the data for homology, the 5' end of the alignment (nucleotides 1 to 148 [*E. coli*

nomenclature]) including the hypervariable V1 region was removed during editing, the region from nucleotide 1478 to the 3' terminus including variable region V5 was also deleted, and small changes were made manually when it was evident that the gaps were misplaced. No other variable regions over the 1,350-nucleotide length were removed. Distance and homology matrices were generated, and representative values are shown in Table 3. A phylogenetic tree was drawn by the neighbor-joining method with *A. nidulans* as the outgroup to root the tree (Fig. 2).

The 16S rRNA sequences from *G. haemolysans* and *G. morbillorum* showed high sequence similarity (98.4%), and the gemellae formed a phylogenetically distinct branch in the tree separate from the streptococcal cluster. The enterococcus sequences showed the highest degree (ca. 86.4%) of homology with the sequences of the gemellae, and *Pediococcus parvulus* demonstrated the least degree (77.5%). The 18 streptococcal 16S rRNA sequences were 83.5 to 85.6% homologous with the *Gemella* sequences, while the sequences within the streptococcal group showed 93.0 to 99.6% homology with each other. In the sequence alignment, the streptococcal sequences also showed a unique pattern at position 234 (CT), position 845 (GGG), and position 1264

(CG) (*E. coli* nomenclature). The other sequences, including those of *G. morbillorum* and *G. haemolysans*, do not contain these nucleotide patterns. *G. haemolysans* and *G. morbillorum* possess an exclusive signature at positions 667 and 709; an adenine replaced an otherwise conserved guanine and a thymine (uracil), respectively. These sequence signatures may be useful in designing genus-specific probes.

We have shown through 16S rDNA sequence comparisons and DNA-DNA hybridizations that *G. haemolysans* and *G. morbillorum* are separate species in the genus *Gemella* and that the gemellae are phylogenetically distinct from the streptococci and other gram-positive bacteria with low G+C contents. The phylogenetic tree presented here was based on a continuous region of 1,350 nucleotides of the 16S rDNA sequence. Other trees constructed from alignments in which one or more internal variable regions were removed changed the branching order and some branch lengths but did not alter the placement of the gemellae on a distinct branch (data not shown). In addition, in every tree configuration, organisms formed tight clusters according to genus, in agreement with previous taxonomic classification.

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