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M or M-Like Protein Gene Polymorphisms in Human Group G Streptococci

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Many group G streptococci (GGS) isolated from infected humans (but not from animal sources) express M or M-like proteins with biological, immunochemical, and genetic features similar to those of group A streptococci (GAS). To further elucidate the recently proposed M-like protein gene (*emmL* gene) polymorphisms in GGS, Southern blots of genomic DNAs from 38 epidemiologically unrelated GGS strains isolated from human specimens and 12 GGS strains recovered from animal sources were hybridized with oligonucleotide probes designed to specifically detect GAS M class I and M class II M protein (*emm*) genes. All human-associated GGS strains showed DNA homology to the GAS M class I *emm* gene probe, whereas no hybridization was found with DNA from any of the animal-associated strains. The *emmL* genes from all human isolates were amplified by PCR, and the complete sequence of the *emmL* gene of the Rebecca Lancefield grouping strain D166B was determined. Again, this gene exhibited the structural features typical for *emm* genes of M class I GAS. The 5′ regions of the PCR-amplified *emmL* genes of the remaining 37 human GGS strains were sequenced. This region showed a sequence diversity similar to that known for GAS *emm* genes. When strains whose N-terminal *emmL* gene sequences showed a homology of >95% were defined as belonging to one genetic type, 30 strains were segregated into six distinct genetic types, whereas the remaining 8 strains each exhibited a unique *emmL* gene sequence. A high degree of homology between the N-terminal *emmL* gene segments of six GGS strains and the corresponding regions of either the *emm*12 or the *emm*57 gene of GAS was found, suggesting a horizontal gene transfer between strains of these species of beta-hemolytic streptococci. Besides a further understanding of the evolution of GGS *emmL* genes, the observed *emmL* gene polymorphisms in GGS could provide the basis for a molecular subspecies delineation of strains and offers the potential of typing GGS for epidemiological purposes.

The widespread distribution of large-colony-forming group G beta-hemolytic streptococci (GGS) as commensals and pathogens in domestic animals has long been recognized. In humans, GGS may colonize the pharynx, skin, large intestine, and genital tract. In recent years, they have been reported with increasing frequency to cause a wide variety of human infections such as pharyngitis, lower respiratory tract infections, cellulitis, sepsis, meningitis, and endocarditis (4, 6, 13, 24, 27, 30, 42, 46). Of note, as with group A streptococci (GAS), pharyngitis due to GGS may be complicated by acute glomerulonephritis or reactive arthritis (12, 16, 17, 37, 39, 49). However, the development of acute rheumatic fever as a nonsuppurative sequela has never been observed.

Human- but not animal-associated strains of GGS and GAS can share virulence mechanisms, including the expression of streptokinase, hyaluronidase (7), C5a peptidase (10), and M protein (3, 4, 11, 19, 44). The M protein is thought to be a primary virulence factor because this cell surface-associated protein confers upon streptococci the ability to resist phagocytosis by polymorphonuclear leukocytes.

M proteins of GAS exhibit a substantial polymorphism, leading to about 80 serologically defined M types. This polymorphism is known to be due to a high degree of heterogeneity among the N termini of diverse M proteins, whereas the cell wall-associated proline-glycine-threonine-serine (PGTS)-rich domains and the leader peptides are conserved among GAS M proteins and also share structural features with a large number of proteins of other gram-positive bacteria (22). The different GAS serotypes are associated with two major M protein classes (1). Sequence data for the PGTS-rich domain of GAS M proteins give evidence for the presence of different types of PGTS-rich domains. Among these, one is specific for class I M proteins (PGTS type I), while the other is specific for class II M proteins (PGTS type IIa) (1, 2).

Expression of either class I or class II M proteins has been found to be associated with several pathogenic properties of GAS (1). Moreover, only isolates expressing class II M proteins produce serum opacity factor (OF) (1), an apolipoproteinase (29) with unknown association to virulence.

M proteins of GAS and GGS recovered from human infections have similar biological, immunochemical, and structural features (3–5, 11, 19, 25, 28, 44). Recently, Collins et al. (11) published the first complete sequence of an M protein gene (*emm* gene) of a human GGS strain. The M protein encoded by this *emm* gene exhibited structural features analogous to those of class I M proteins of GAS. On the basis of hybridization profiles, the authors deduced the existence of at least four different *emm* alleles associated with GGS. Smirnov et al. (45) published the sequence of a GGS gene encoding an immunoglobulin G-binding protein. The deduced amino acid sequence exhibited characteristic features of a class I M protein but differed significantly in its N-terminal sequence from the corresponding segment of the GGS M protein described by Collins et al. (11). Therefore, the available data suggest that, like GAS, GGS can harbor various *emm* or *emm*-like (*emmL*)

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TABLE 1. GGS strains of human origin investigated in the present study

Strain designation		Isolation site and/or clinical disease[c]	Source[d]
Aachen[a]	Original[b]		
444	74-530	Throat, pharyngitis	S
453	Börecki MN	Clinical isolate	S
455	S.U.N.Y. MN	Clinical isolate	S
456	Ragin MN	Clinical isolate	S
461	B 981 MN	Clinical isolate	S
480	G 1167	Skin infection	B (31)
481	G 148	Clinical isolate	B (31)
484	G 120	Skin infection	B (31)
485	G 1400	Skin infection	B (31)
487	GSHS 13	Skin infection	B (31)
490	G 493	Clinical isolate	B (31)
492	G 88	Clinical isolate	B (31)
494	G 10134	Clinical isolate	B (31)
496	G 7809	Clinical isolate	B (14, 31)
497	G 7805	Clinical isolate	B (14, 31)
500	G 78	Clinical isolate	B (31)
503	G 60	Clinical isolate	B (31)
507	Va II 60457	Perianal abscess	A
510	Va II 65869	Skin infection	A
593	D166B	Epidermolysis bullosa	L
643	CS 121	Throat, pharyngitis	C
652	CS 241	Skin infection	C
653	CS 242	Throat	C
654	CS 243	Skin infection	C
655	CS 244	Throat, pharyngitis	C
656	CS 245	Skin infection	C
657	CS 246	Skin infection, acute glomerulonephritis	C
658	CS 247	Skin infection	C
724	Va II 21572	Skin infection	A
801	Va II 94182	Skin infection	A
802	Va I 94457	Throat, pharyngitis	A
861	Va I 1961	Middle ear fluid	A
934	Va II 11485	Vagina, colpitis	A
935	Va II 12066	Skin infection	A
936	Va I 11251	Middle ear fluid	A
950	Va II 69135	Skin infection	A
953	Va I o. N.	Throat, pharyngitis	A
971	Va II 17838	Skin infection	A

[a] Numbers that were given to the strains in our laboratory and that are used in the present communication.

[b] Original designations in the laboratories from which the strains were obtained.

[c] The majority of strains were isolated from patients with clinically relevant infections. The strains merely described as clinical isolates were isolated from various clinical specimens, but no further information regarding the site of isolation or the clinical significance was available.

[d] A, Clinical microbiology laboratory, Technical University Aachen, Aachen, Germany. B, M. D. P. Boyle, Medical College of Ohio. C, P. P. Cleary, University of Minnesota. L, R. Lancefield, Rockefeller University, New York, N.Y. S, P. M. Schlievert, University of Minnesota. Some of the strains were investigated in previously published studies (references in parentheses).

genes. Given that the nomenclature of *emm* and *emmL* genes is currently a subject of debate, for reasons of convenience the GGS genes are simply referred to as *emmL* genes in this paper.

In the present study, we analyzed a large number of epidemiologically unrelated GGS strains for the presence of *emmL* genes; we describe a PCR assay which reliably amplified *emmL* genes from each human GGS strain tested, whereas none was found in any of the strains isolated from animal sources. Results from phagocytosis assays could be correlated to these findings. On the basis of data from hybridization experiments and from the sequence of a new entire *emmL* gene, we provide further evidence that all human GGS tested harbor *emmL* sequences which are associated with the class I *emm* gene lineage of GAS. In addition, extensive sequence homology between the 5′ regions of *emmL* genes from GAS and GGS strains was found. Moreover, it is shown that the 5′ regions of *emmL* genes from human GGS display a substantial diversity and that this gene polymorphism in GGS could provide the basis for a subspecies delineation of strains.

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MATERIALS AND METHODS

Bacterial strains. Thirty-eight GGS strains isolated from human specimens were obtained from P. P. Cleary and P. M. Schlievert (University of Minnesota, Minneapolis) and M. D. P. Boyle (Medical College of Ohio, Toledo) or were collected in the clinical microbiology laboratory of our institution (Table 1). Strain 593 is identical to the Rebecca Lancefield grouping strain D166B (ATCC 12394). The human GGS strains were isolated from 1965 to 1993. An additional 12 GGS strains isolated from animal sources (provided by P. P. Cleary) were included in this study. When grown overnight on agar plates supplemented with 5% sheep blood in a CO_2-enriched atmosphere, all isolates exhibited typical zones of beta-hemolysis and large-colony morphology. They were confirmed as GGS by identification of their group carbohydrate antigen with a commercially available latex agglutination test (Streptex, Dartford, England), and biochemical and enzymatic characteristics were determined with the ApiStrep system (bioMerieux, La Balmes-les-Grottes, France). Thus, the organisms investigated in this study were definitively distinguished from small-colony-forming beta-hemolytic streptococci that also may exhibit the Lancefield group G cell wall carbohydrate antigen but taxonomically belong to a different species (*Streptococcus anginosus*, or "*Streptococcus milleri*" group). The strains were stored at −70°C in brain heart infusion broth (Unipath, Wesel, Germany) supplemented with horse serum.

Assay of resistance to phagocytosis. The ability of the GGS strains to resist phagocytosis was tested according to a slight modification of the protocol of Lancefield (23). GGS strains were grown for 18 h (37°C, 10% CO_2) in Todd Hewitt broth (Oxoid, Basingstoke, England) supplemented with 5 g of yeast extract (Gibco, Pasley, Scotland) per liter (THYB). After this incubation, a 0.1-ml volume was transferred into 5 ml of prewarmed THYB and incubated (37°C, 10% CO_2) for precisely 90 min. Subsequently, 10-fold dilutions were prepared in phosphate-buffered saline (pH 7.4) and further diluted 1:4 to provide 50 to 100 CFU/0.1 ml of sample. The number of CFU (zero count) was determined by streaking 0.1-ml samples onto sheep blood agar plates. A total of 100 μl from the 10^{-4} dilution step was added to 0.3 ml of heparinized (25 IU/ml) human blood from healthy blood donors. The suspension was incubated at 37°C for 3 h while being rotated end over end eight times per minute in sterile capped polystyrene tubes. The number of CFU after this incubation was determined as described for the zero counts. After overnight incubation at 37°C, any colonies present were counted and subsequently picked from the 3-h plates to be used for two repetitions of the entire test.

The M protein-negative GAS strain T11-30/50, which was obtained from the Czechoslovak National Collection of Type Cultures, was taken as the negative control. Five GAS strains of different M types (types 1, 2, 6, 12, and 49) were used as positive controls.

Expression of OF. The production of streptococcal lipoproteinase by each individual strain was tested according to the protocol of Maxted et al. (29).

Nucleic acid techniques. Streptococcal genomic DNA was prepared by a slight modification of the protocol of Martin et al. (27) from 10-ml bacterial cultures grown in THYB. The DNA was subjected to an additional purification step involving a twofold phenol-chloroform extraction. Finally, the precipitated DNA was dissolved in TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA) containing 1 μg of RNase A (Sigma, Deisenhofen, Germany) per ml. Ten-microgram aliquots of DNA were digested with the restriction enzyme *Xba*I (Boehringer, Mannheim, Germany) by following the manufacturer's instructions. PCR was performed with a TRIO-TB-1 thermal cycler (Biometra, Göttingen, Germany) and included 27 cycles; each cycle consisted of 1 min at 95°C, 1 min at 55°C, and 2.5 min at 72°C. Reaction mixture volumes were 100 μl and contained PCR buffer (50 mM KCl, 10 mM Tris [pH 7.5], 1.5 mM $MgCl_2$, 0.01% gelatin), 200 μM each deoxynucleoside triphosphate, 0.5 μM each primer, 1 μg of template DNA, and 2.5 U of *Taq* polymerase (Gibco BRL, Eggenstein, Germany). The *emmL* gene of strain 593 was amplified with the "all M" primers described by Podbielski et al. (35) (Table 2), whereas the remainder of the *emmL* genes were amplified with primers specifically designed for GGS *emmL* genes (G1F and G1R [Table 2]). Both the restriction fragments of genomic DNA and the PCR products were subjected to agarose gel electrophoresis and subsequently Southern blotted onto Biodyne B membranes (Pall BioSupport, Dreieich, Germany) by conventional methods (40). Sequencing of the complete *emmL* gene of the reference strain 593 was performed according to the method of Sanger et al. (41) with the set of primers listed in Table 2. By utilizing the PCR products as templates and G1F as

TABLE 2. Oligonucleotides used in this study

Oligonucleotide designation[a]	Sequence (5′ to 3′)
A	
G1F	AAA AAT AAG GAG AAA AAA TGG
G1R	TTT TTA GTT TTC TTC TTT GCG
allM-forward[b]	GGG GGG GGA TCC ATA AGG AGC ATA AAA ATG GCT
allM-reverse[b]	GGG GGG GAA TTC AGC TTA GTT TTC TTC TTT GCG
B	
PGTS I[c]	TCC AGG TAA AGG TCA AGC AC
PGTS IIa[c]	GAT CAG CAA TGA CGC AAC A
C	
a	ATC TAG TAC TTC ATT TTT AGC TTC GGT ATA
b	ATT TCT AGC AGC GGT ATA GTT AGG GTG AGT
c	AAT AGG CAT CAT CGA AGT AAT ATC TTC ACT
d	GTC TGC TCT AGC TGT ATC ATA TGT AGG ATG
e[d]	ACG TTG TTT TTC TGC GAC TAA ATC ACT ATG
f	TTC TTC TCT GAT TTT CTC AAT ATC AAC ACT
D	
G1F	AAA AAT AAG GAG AAA AAA TGG
G1R	TTT TTA GTT TTC TTC TTT GCG
GGS-BEA09	CTT CAG CTT GTT TCG CTA AT
GGS-BEA10	ATT AGC GAA ACA AGC TGA AG
GGS-BEA17	AAG AAC GTC TTC GTA AGG AA
GGS-BEA18	CGA ACT AGT TCG TCA AGT TG
GGS-BEA21	CGA AGA CCT ACG GCC TTG CT
GGS-SIG13	GAA GAG CAG CTA ATG TGC TG
GGS-SIG14	AGA AGA AGC AAA CAG AAC AT

[a] Group A oligonucleotides were used to generate PCR products. Group B oligonucleotides were probes directed against M class-specifying regions of *emm* genes of GAS. Group C oligonucleotides were probes specifically directed against N-terminal regions of *emmL* gene types a to f. Group D oligonucleotides were used to sequence the *emmL*G593 gene.

[b] Sequence previously published by Podbielski et al. (35).

[c] Sequence previously published by Bessen et al. (2).

[d] Sequence previously published by Robbins et al. (38).

a sequencing primer, the sequences of the 5′ regions of the *emmL* genes of every other GGS strain were determined with an automated model 373A DNA sequencer and the PRISM sequencing kit (Applied Biosystems, Weiterstadt, Germany) as outlined in the manufacturer's instructions. Sequences were assembled and analyzed with the aid of PC GENE software (IntelliGenetics, Mountain View, Calif.).

Sequences (all 30 nucleotides in length) that were complementary to the coding strands of parts of the N termini encoding regions of the GGS *emmL* genes were selected as probes (Table 2). All oligonucleotides were prepared on an Oligo 1000 DNA synthesizer (Beckman Instruments, Munich, Germany).

Purification and digoxigenin-dUTP labeling of the oligonucleotides and hybridization assays were performed as previously described in detail (21). For visualization of hybridization reactions, the chemiluminescent substrate 3-(2′-spiroadamantan)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetan (Boehringer) was used in accordance with the instructions of the manufacturer, and light signals were detected by exposure of the membranes to x-ray film (Cronex 4; Du Pont, Bad Homburg, Germany).

Nucleotide sequence accession numbers. The nucleotide sequences of *emmL*G593, *emmL*G485, *emmL*G480, *emmL*G861, *emmL*G656, *emmL*G643, *emmL*G487, *emmL*G494, *emmL*G496, *emmL*G500, *emmL*G507, *emmL*G652, *emmL*G653, *emmL*G953, and *emmL*G935 have been submitted to the EMBL data library and have been assigned accession numbers X60098, X79521, X79520, X79532, X79529, X79528, X79522, X79523, X79525, X79526, X79527, X79530, X79531, X79534, and X79533, respectively.

RESULTS

Assay of resistance to phagocytosis. All M protein-positive GAS control strains and 9 of the 14 human-associated GGS strains tested were found to resist phagocytosis, as the increases of colony counts after 3 h of rotation in human blood were greater than 32-fold of the original bacterial inoculum (23). One additional human GGS strain was also able to multiply in human blood, but to a lesser degree (strain 507 [Table 3]). In contrast, the remaining four human-associated GGS, all of the animal-associated GGS strains tested, and the M protein-negative GAS control strain did not resist phagocytosis by polymorphonuclear leukocytes (fold increase, <1 [Table 3]). No correlation between the site of strain isolation and the extent of resistance to phagocytosis was seen. The results of the phagocytosis inhibition assay were in good agreement with previous observations which indicated that most human GGS strains resist phagocytosis and thus are believed to be producers of M protein (3–5, 11, 19, 25, 28, 44), whereas animal isolates consistently do not present this phenotype (44).

Human GGS strains contain DNA homologous to that of M class I *emm* genes. When tested for the expression of OF, all human and animal strains under study were found to be OF negative. In order to determine whether this phenomenon was correlated with PGTS-rich domain type I encoding *emmL* gene sequences, we analyzed the *Xba*I-digested genomic DNA from all human and animal GGS strains by hybridizations with PGTS I- or PGTS IIa-specific oligonucleotide probes in Southern blot experiments.

No hybridization with either probe was detected with DNA from any of the animal-associated GGS strains. In contrast, all strains isolated from human specimens showed specific hybridization signals with the PGTS I- but not with the PGTS IIa-specific oligonucleotide probe. These results clearly indicated that all investigated human isolates of GGS harbor sequences homologous to segments regarded as specific for class I *emm* genes of GAS.

PCR assay for amplification of *emm*-like genes in human GGS isolates. The PCR primers G1F and G1R (Table 2) were designed on the basis of the *emm*G1 (11) nucleotide sequence. When these primers were applied, no specific PCR amplicon was generated with template DNA from the 12 animal isolates, whereas *emmL* DNA fragments from every human GGS isolate tested were successfully amplified. The sizes of the resulting PCR products ranged from about 0.95 kb (strain 492) to approximately 1.8 kb (strains 802 and 936), a size range similar

TABLE 3. Assay of resistance to phagocytosis

Strains investigated	Fold increase in numbers of colony counts after 3 h
M protein-positive GAS strains[a] ($n = 5$)	>100[b] (for all strains)
M protein-negative GAS strain[c]	<1
Animal-associated GGS strains ($n = 5$)	<1 (for all strains)
Human-associated GGS strains	
480	>100
487	>100
494	60
496	>100
500	<1
503	34
507	8
643	<1
652	<1
653	54
654	<1
656	>100
935	41
936	38

[a] Positive controls, GAS strains of M types 1, 2, 6, 12, and 49.
[b] A fold increase of >100 indicates innumerable colonies after 3 h, with blood completely hemolyzed.
[c] Negative control, M-negative GAS strain T11-30/50.

to that known for GAS *emm* genes. In Southern blot hybridization experiments, again all PCR products specifically hybridized with the PGTS I-specific oligonucleotide probe.

Nucleotide sequence of the *emm*-like gene from strain 593 (*emmL*G593). PCR with DNA from strain 593 as the template was performed as described above. The resulting product was directly sequenced. Upon analysis of the assembled sequence, an open reading frame of 1,326 bp encoding a deduced polypeptide of 441 amino acids (aa) was identified. We termed the gene *emmL*G593 and the deduced protein EmmLG593 (Fig. 1).

When the EmmLG593 sequence was analyzed, a signal peptide potentially cleaved C terminal to residue 41 was predicted by the computer program. The 41 residues of the putative signal peptide showed 90, 88, and 75% sequence identity with the signal peptides of GGS proteins MG1 (11) and FcRV (45) and GAS type 12 M protein (38), respectively. A second region of extensive homology spanned the region between aa residue 235 and the carboxy terminus. Like any other M or M-like protein, EmmLG593 harbors typical C repeats within this region, consisting of three 23-aa repeat units (designated CR1 to CR3 in the sequence depicted in Fig. 1) that are joined by different spacer sequences (termed SE1 and SE2 in Fig. 1) in between. These C repeats were almost identical to those known for MG1 and FcRV or GAS class I M proteins. Like *emm*G1 and *fcrV*, *emmL*G593 did not contain prominent tandem repeats comparable to the so-called A and B repeats located in the central portions of some GAS *emm* genes.

The DNA sequence encoding the N terminus of the mature EmmLG593 protein showed only limited homology to the corresponding regions of other M- or M-like proteins. This variable region is known to encode the specific epitopes for M-typing antisera in GAS *emm* genes. As shown in Fig. 1, upstream from *emmL*G593, a region of 221 bp including the putative promoter sequences was also determined and was found to be highly homologous to the corresponding regions of the *emm*G1 (11) and the *fcrV* (45) genes, whereas homologies to corresponding GAS *emm* gene sequences reached only 60% (32, 36).

The region of EmmLG593 downstream of the C repeats comprised a PGTS-rich domain typically found in M-like proteins and showed 97 and 96% sequence homology to the corresponding C-terminal regions of MG1 and FcRV, respectively.

As an example, the amino acid sequences of the PGTS-rich domains of several M or M-like proteins of GAS and GGS were aligned (Fig. 2). The PGTS-rich domain of EmmG593 showed ≥95% sequence identity with the type I PGTS-rich domains of GAS M6 and M12 proteins and the PGTS-rich domains of the GGS proteins MG1 and FcRV. Conversely, homology to representatives of the type IIa PGTS-rich domains of GAS (ML2.1 and M49) did not exceed 54%. Thus, sequence data clearly associated EmmG593 to PGTS type I, class I M-like proteins.

***emmL* gene polymorphisms in GGS.** On the basis of the sequences of *emmL*G593, *emm*G1, and *fcrV*, we designed oligonucleotide probes corresponding to the nonhomologous segments of their N-terminal regions. When the PCR products of all GGS *emmL* genes investigated in this study were transferred to nylon blots by the Southern technique and tested with these probes, only the PCR products of the GGS strains 503 and 950 reacted with the *emmL*G593-specific probe, whereas the two other probes did not hybridize to any of the PCR-amplified *emmL* genes (data not shown). These results indicated a variability of this particular *emmL* gene region in GGS.

In order to identify the range of sequence diversity in human GGS *emmL* genes, we sequenced the 5′ ends of PCR-amplified genes from every GGS strain investigated as described above. As expected, the (5′ *emmL*) sequences encoding the N termini of the mature M-like proteins exhibited considerable sequence variability (Fig. 3) comparable to those known for the corresponding portions of GAS *emm* genes (20, 21). When two sequences were defined as belonging to the same GGS *emmL* genotype, if they demonstrated >95% homology within the 120 bp encoding the mature N termini, the 38 GGS *emmL* genes could be associated to 14 GGS *emmL* gene types, arbitrarily designated types a to n (Table 4). Within the defined region, type-to-type homology reached a maximum of only 52% (types c and h). When the corresponding *emm*G1 and *fcrV* sequences were subjected to the same analysis, they were found not to belong to any of the 14 genotypes (Fig. 3). There was no correlation between a given GGS *emmL* gene type, the site of strain isolation, or the degree of resistance to phagocytosis. These results were in good agreement with the observations of Martin et al. (27), who found no correlation between growth in human blood and the DNA fingerprint patterns of several GGS strains.

Homology between the N termini encoding *emm* or *emmL* sequences of GAS and GGS. When the GGS *emmL* gene sequences were compared with published GAS *emm* or *emmL* sequences (compiled in references 33–35 and 48), gene portions of *emmL*G480 and *emmL*G656 were found to exhibit >85% homology to the GAS *emm*57 (26) and *emm*12 (38) sequences (Fig. 4). The GGS *emmL* gene sequence of strain 656, representing GGS *emmL* gene type e, and the GAS *emm*12 gene sequence exhibited nearly complete identity beginning at bp 100 (Fig. 4A). Of note, the GGS strains 656 and 658 are both known to express M12 antigen (8). Additionally, the GGS *emmL* gene sequence of strain 480, which is GGS *emmL* gene type c, and the GAS *emm* gene sequence of an M type 57 strain showed a homology of 85% within the entire region aligned for maximum homology (Fig. 4B).

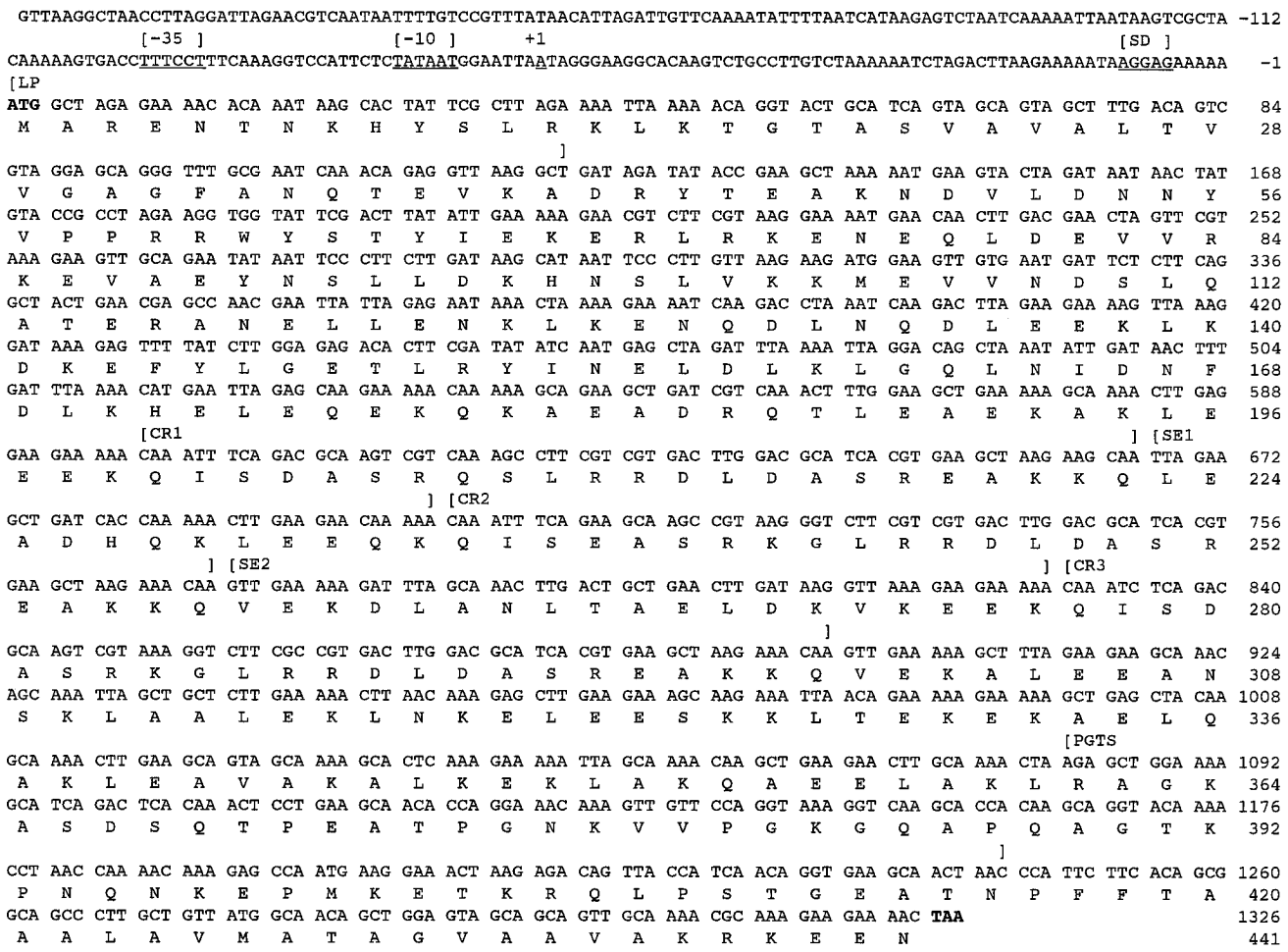

FIG. 1. Nucleotide sequence of the *emmL* promoter region and the *emmL* gene of GGS strain 593 (*emmL*G593). The deduced amino acid sequence is shown under the corresponding codons. The start and stop codons are in boldface. Special features within the sequence, such as the gene regions encoding the leader peptide (LP), the C repeats (CR1 to -3), the spacer elements (SE1 and SE2), and the sequence encoding the PGTS-rich domain, are marked by brackets above their first and last nucleotides. Upstream of the *emmL*G593 sequence, a potential Shine-Dalgarno box (SD), an experimentally assessed transcription start site (+1) (32), and the deduced −10 and −35 boxes are underlined.

DISCUSSION

The main objective of the present investigation was to analyze a considerable number of human- and animal-associated GGS strains to address the following topics: (i) the ubiquitous occurrence of *emmL* genes in human GGS isolates; (ii) the existence of an *emmL* gene polymorphism in GGS, which could provide the basis for an "oligotyping" system; and (iii) the indication for exchanges of *emmL* gene sequences between GAS and GGS.

Within the last few years, molecular genetic techniques became important methods to identify and characterize *emm* or *emm*-like genes of GAS (9, 15, 18, 20, 21, 34, 35). Thus far, however, identification of M or M-like proteins in GGS is primarily based on functional and serological analysis (7, 25, 28, 31), and only a few GGS *emm* or *emmL* genes were studied by molecular means (3, 5, 11, 45). Therefore, the development of the PCR assay established in this study that allows probably universal amplification of *emm* or *emmL* genes from these bacteria could certainly accelerate their investigation.

Most of the human GGS strains under study were capable of multiplying in heparinized blood, whereas all animal-associated strains were not. On the basis of the concept that the expression of M protein is the factor that is mainly responsible for conferring upon streptococci the ability to resist phagocytosis, these data are in accordance with our findings that the presence of *emmL* genes was a unique feature of GGS strains isolated from human specimens. This confirms the observations of Simpson et al. (44), who also found *emm* gene-related DNA sequences only in human-associated GGS strains when testing 8 human- and 12 animal-associated GGS strains in hybridization experiments. Together with other findings, such as the biochemical differences between human and bovine GGS (7), the lack of *scpA* genes in animal-associated GGS (10), and differences in immunoglobulin-binding proteins (31), the available data suggest that GGS of human and animal origins could be distinct taxonomic entities. Further studies to determine whether this is true are currently in progress in our laboratory.

The *emmL* gene sequences of all human isolates tested hybridized exclusively with a PGTS type-I-specific oligonucleotide probe which is strongly associated with M class I protein in GAS (1, 2). These results were supported by the sequence data for the *emmL*G593 gene presented in this report (Fig. 1) as well as by the sequences of the *emm*G1 (11) and *fcrV* (45)

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M (-like) protein                                                      PGTS-class

       M12         RAGKASDSQTPEAKPGNKaVPGKGQAPQAGTKPNQNKEPMKETKR*QLPSTGEATN     I
       M6          RAGKASDSQTPEAKPGNKVVPGKGQAPQAGTKPNQNKEPMKETKR*QLPSTGEATN     I
                   ::::::::::::: :::::::::::::::::::::::::::::: ::::::::::
       EmmLG593    RAGKASDSQTPEATPGNKVVPGKGQAPQAGTKPNQNKEPMKETKR*QLPSTGEATN
       FcRV        RAGKASDSrTPEATPrNKVVPsKGQAPQAGTKPNQNKEPMKETKR*QLPSTGEATN
       MG1         RAGKASDSQTPdAkPGNnVVPGKGQAPQAGTKPNQNKEPMKETKR*QLPSTGEATN
                      :    ::: ::             :       : :      :  ::::::  :
       ML2.1          K**GNQTPNAKVAPQANRSR*SAMT******QQK*******RT*LPSTGETAN    IIa
       M49            K**GNQTPNAKVAPQANRSR*SAMT******QQK*******RT*LPSTGETAN    IIa
```

FIG. 2. Amino acid alignments of the PGTS-rich domains of selected M and M-like proteins of GAS (ML2.1, M6, M12, and M49) and GGS (EMMLG593, FcRV, and MG1). The definition of PGTS-rich domains and the designation of classes are according to those of Bessen and Fischetti (2). Sequences are aligned for maximum homology. Identical positions in sequences of different PGTS classes are indicated by colons; asterisks indicate a missing amino acid at the position. Residues differing from the consensus sequence of each PGTS class are in lowercase type. Nucleotide and amino acid positions are as follows: M12 (bp 2765 to 2929) (38), M6 (aa 361 to 415) (15), EmmLG593 (bp 1093 to 1257) (this study), FcrV (aa 507 to 561) (45), MG1 (bp 1848 to 2012) (11), ML2.1 (aa 306 to 341) (2), and M49 (bp 1013 to 1120) (18).

genes, which consistently exhibited structural features characteristic for M class I *emm* genes. Thus, all human GGS strains examined thus far harbor structurally related *emmL* genes, which provides good evidence that, with respect to their conserved regions, GGS *emmL* genes could be offspring of one common ancestor gene. This assumption is consistent with the OF-negative phenotype of GGS, which is also a property typical of GAS strains expressing class I M proteins. The overall uniformity of the conserved portions of GGS *emm* or *emmL* gene sequences made it possible to design a single pair of PCR primers to reliably amplify GGS *emmL* genes. This technique facilitated the generation of *emmL* gene products from every human GGS strain tested in a unique PCR assay, a situation which is even more convenient than with GAS. Thus, the straightforward approach established in the present study could have a substantial impact on further functional and epidemiological studies of these genes.

The observed size variations of the *emmL* gene PCR products, along with results from hybridization experiments (3, 11, 44; also this study), and sequence analysis of *emmL*G593 (this study), *emm*G1 (11), and *fcrV* (45) suggested a polymorphism in GGS *emmL* genes due to a diversity within the 5′ ends of these genes. To reveal the extent of this polymorphism, we sequenced the N-terminal portions of 37 additional human GGS *emmL* genes.

The sequence data (Fig. 3) showed N-terminal *emmL* gene

```
                                                                                                              bp
                                                 >
a      GTAGGAGCAG GGTTTGCGAA TCAAACAGAG GTTAAGGCTG ATAGATATAC CGAAGCTAAA AATGAAGTAC TAGATAATAA CTATGTACCG CCTAGAAGGT   184
b      ---------- ----A--A-G C--G-----A ---------- -GGTTAC-CA -CCTAACT-T -CC-CT-CTA G-A--G-AGT ACTAAG-GAT -TGTATGTA-
c      ---------- -A--A-TAGC AGGGCAGACA --A-----CA ---ATAG-GA A--TAT--CT TCGATGA-G- CTAT-TTG-G TGG---TGGT T-A--T-ATG
d      T----G---- -A--A--A-C C--------A --------G- -G-ATA--CA TCCTA-AT-T G--AC--CTA G--CAG-CGC --TGCG-GAA -T-G--TATG
e      T----G---- -CC-G-TAGC AGGGCAGACA --A-GA--A- --CATAG-GA TTT--TCGC- G-AA--CA-- GTTTAG-AG- T-TA-G--AA AAATTTGAAA
f      -GG-C--G-T TAGCG-TT-- C--------A --------G- -G--TGT-GA TATT-AG--- -TCAG--A-G A--CCTTA-- AG-A--TATT GGC----T-G
g      -GG-C--G-T TAGCG--T-- C---C----A --------G- CGGAGA---- AT-C-A--GA TGGAGGAG-A C---AG-GGC TAGAC--TAC -TAGTG----
h      ---------- -A--A-TAGC AGCGCAGACA --A-----CA ---ATAG-GA A--TAT--CT TCGATGA-G- CTAT-TTG-G TGG---TG-T -ACGT-T-CA
i      ---------- ----A--ACG C--G-----A --------G- --CATCCGCG A-T--AA--C GC-AG-CATG AN-TGCTA-C N---CACTAT GTACCGGCTG
j      -GG--G---- -A--A--ACG C--------A ----G---G- TGGATA--G- A-CTCAAT-C -GGC--T-GA C--GAG-AG- GC--AG-ACT GA-GAGCTAG
k      -GG-C--GGT TAG---TC-- -ACT-AT--A ----GT--A- CCGTGAC--G GTCTAAG-C- G----TCCGG A-A-A-TGC- AA-AC-TATT TT-GACCTTG
l      T----G---- -A--A--A-C C--------A --------G- -G-ATG-ACA -CCTCGCT-T -CC-CT-CTA G-A--G-AGT ACTAGA-TAT AA-TATGTAC
m      ---------- ----A-TA-C AGGGCAGACA --A-GA---- --CA-A--CA A-TTGA-TT- ----CTCA-G C--C---A-T G-GGAAG-AA AAAGTTTTAG
n      -----G---- -A--A-GA-C C--------A --------G- GGGAG--CT- ATCCTAC-TT TTAT-CAGGT ACAGC-GACG -CTGC--TAC TTGGAT-TG-
emmG1  T----G---- -A--A--A-G C--------A ---------- -A-ATAC-GA -C--TA---T C-GT-T--TA A-A-G-C-G- AG-A-AGAG- A--C----T-
fcrV   T----G---- -AC-A--A-G C-----T--A --------G- -AGAGA--GA GAG-TTA-G- C-A-CCAA-G A-C-AGC-TT AC-A-AGGTT AT-GACCAAA

a      GGTATTCGAC TTATATTGAA AAAGAACGTC TTCGTAAGGA AAATGAACAA CTTGACGAAC TAGTTCGTAA                                   254
b      C-GG-AAT-T -CGGGAGTGG ---A-T-AA- GAAT--CC-- -CTAC-GTC- ---TCA-GTA A-ACAATCTT
c      CAGT-GATT- -A-CT--ACT GCG--T-AA- --GC----AG --TGA-TG-T -CGA-A-
d      TACC-AGTGA C-TATGGCTT C-NC-GTA-G AAAAA--TC- GG-GTT-ACT AAAA-AATGA GGAG-TACCT
e      -ACTGAAACA GCG-CCA--- CTCT-C-T-- AG-AAT-CT- TG--A-TAA- TCAACT-G-T ATAACG--G-
f      AC---GGTCA ACTA-G-A-T -CTTT-GC-G GATCATTCCG TG-GA-TAGT GCGTTAA--G A-ACCAT---
g      T-CGA--TGA GC-C---TCT -CT--GA-AT A-ACACTCAG -C-AAG---G T-GAG-T-TG ATTG-TT-GC
h      -TGCA--T-- --TAC-GCGG -TCACTGC-A GAGA-G-T-- CCGACGCATG AAACTATC-G -GTGGGT--G
i      CACGG-G-TT CG-CTCATGT G-CA-C-ACG C-----G-A- -C--AC--TG ACGACTAGT- GCAAG-C
j      AAG-AAAAGA AA-G--A-TT -C---CGCAT C-GC--C--T TGCC--GATT AGCA----G- ---AGACAGC
k      A-GTGGAA-A AC---AGTT- ---ATG-AGA A-GA-G-CTT ----TTTA-- AA-A-A-TGT --AACGA---
l      C-GC-CA-CT -NGGGACC-G T-TA-TAAAA ANAT---A-- -CTA---G-- GCAT-TA--G CTCAAGAAG-
m      AACGAGAAG- AG-ACGA--C -CGTT-GCAG AAGA-TTAAC T-NAA-G--- -AG---T-TG ATT-GGTGGT
n      ACCTAGT--- --GTGGGCTT C-C--GTA-G ACTCANGTC- G-GGATTT-C -----G-GTG ACTCGT--GC
emmG1  TAA--AATGA --TACAAA-T CT-A--GTCA GAGT-CGTC- GCT-C--TCT T-GATG--TG A-C-GGAA-G
fcrV   T-CCC-ATGA -C--C-AA-- --TACTTTAG C-G-GGCATT CCG-A-GA-T -AG--AA-TG A-CGAGA-TT
```

FIG. 3. Alignment of partial nucleotide sequences (5′ to 3′) of GGS *emm* and *emmL* genes. The letters a to n indicate the defined *emmL* gene types. The sequences shown encode the C-terminal segments of the leader peptide and the N-terminal portions of the mature proteins. The indicated base pair positions correspond to the base pair positions of the *emmL*G593 sequence shown in Fig. 1. The first codons of the mature M proteins are marked by an arrowhead. Sequence positions identical to sequence positions in strain 593 (GGS *emmL* gene type a) are indicated by dashes. The sequences to which the type-specific oligonucleotide probes were directed are underlined. The sequences from *emm*G1 and *fcrV* were taken from the works of Collins et al. (11) and Smirnov et al. (45), respectively.

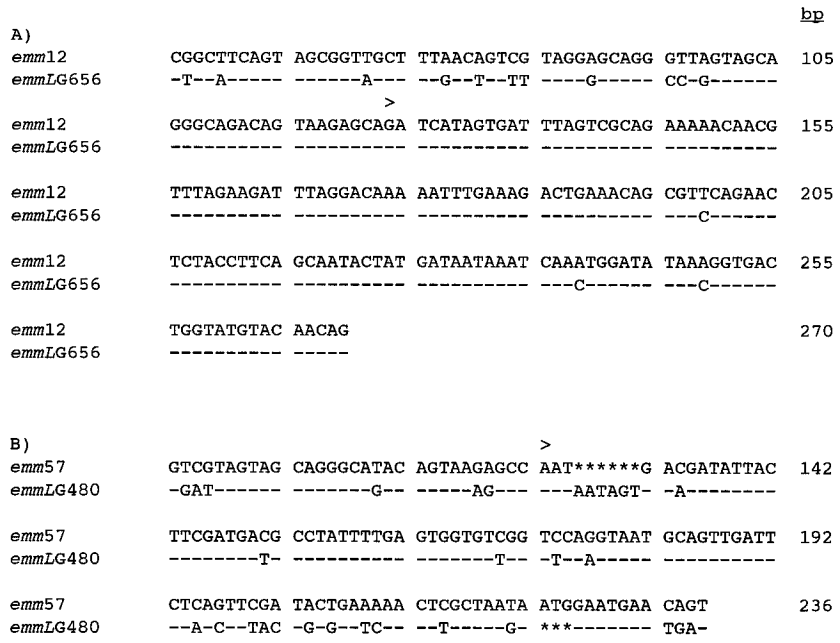

FIG. 4. Alignment of partial nucleotide sequences (5′ to 3′) of the GAS *emm*12 (38) and GGS *emmL*G656 genes (A) and the GAS *emm*57 (26) and GGS *emmL*G480 genes (B). The sequences shown here encode the C-terminal segments of the leader peptide and the N-terminal portions of the mature proteins. The indicated base pair positions are identical to those in Fig. 3. The first codons for the mature M proteins are indicated by arrowheads. The sequences shown in panel B are aligned for maximum homology as indicated by asterisks. Positions in the GGS sequences identical to positions in the GAS sequences are indicated by dashes.

polymorphisms in GGS comparable to that known for GAS and allowed the distribution of the GGS strains into distinct genetic types (Table 4). Preliminary results of experiments performed in our laboratory indicate the possibility of developing a GGS *emmL* gene oligotyping system analogous to that previously established for GAS (21). Further systematic investigations are needed to adapt this approach for typing of GGS as well. From a logistic perspective, this could be accelerated by introducing a reverse dot blot technique in combination with allele-specific nonradioactive oligonucleotide probes, as recently described for the GAS *emm* gene typing (20).

The question of which mechanisms in the phylogenesis of GGS *emmL* genes could have been responsible for the evolution of the allelic variations remains, especially when taking into consideration that nearly identical *emmL* gene sequences are found in GAS and GGS.

Even if there is a formal possibility that *emmL* genes in GAS and GGS independently underwent mutations leading to a convergent development, we think that it is much more likely that there are ongoing horizontal gene transfers and intergenomic recombination events involving movements of partial or entire *emmL* gene sequences between these two species of beta-hemolytic streptococci. Such mechanisms have previously been suggested by Simpson et al. (43).

A possible mechanism for horizontal transmission of *emmL* sequences was suggested by the earlier observations of Totolian (47), who demonstrated that type-specific M antigens could be transduced from a variety of distinct M-type donors to the same GAS recipient strain. Such a mechanism could also be responsible for the development of gene mosaics and the evolution of GAS *emm* and *emmL* genes (33, 48).

Our results shed new light on the polymorphisms and the evolution of genetic changes of a major virulence determinant of human GGS. Moreover, the observed *emmL* gene polymorphisms could provide the basis for a reliable subspecies delineation of human GGS strains that could be beneficial for epidemiologic studies of these increasingly recognized pathogens.

TABLE 4. Segregation of GGS strains into distinct genetic types[a]

Genetic type	Strain(s)
a	503, 593, 950
b	444, 453, 455, 456, 461, 481, 485, 497, 510, 655, 724, 801, 802, 936, 971
c	480, 484, 492
d	654, 861, 934, 935
e	656, 657, 658
f	490, 643
g	487
h	494
i	496
j	500
k	507
l	652
m	653
n	953

[a] Strains exhibiting >95% homology within the first 120 nucleotides of the *emmL* gene region encoding the mature M (or M-like) protein were assigned to one genetic type (arbitrarily designated *emmL* gene types a to n).

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