A Comprehensive Genetic Study of Streptococcal Immunoglobulin A1 Proteases: Evidence for Recombination within and between Species

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An analysis of 13 immunoglobulin A1 (IgA1) protease genes (*iga*) of strains of *Streptococcus pneumoniae*, *Streptococcus oralis*, *Streptococcus mitis*, and *Streptococcus sanguis* was carried out to obtain information on the structure, polymorphism, and phylogeny of this specific protease, which enables bacteria to evade functions of the predominant Ig isotype on mucosal surfaces. The analysis included cloning and sequencing of *iga* genes from *S. oralis* and *S. mitis* biovar 1, sequencing of an additional seven *iga* genes from *S. sanguis* biovars 1 through 4, and restriction fragment length polymorphism (RFLP) analyses of *iga* genes of another 10 strains of *S. mitis* biovar 1 and 6 strains of *S. oralis*. All 13 genes sequenced had the potential of encoding proteins with molecular masses of approximately 200 kDa containing the sequence motif HEMTH and an E residue 20 amino acids downstream, which are characteristic of Zn metalloproteinases. In addition, all had a typical gram-positive cell wall anchor motif, LPNTG, which, in contrast to such motifs in other known streptococcal and staphylococcal proteins, was located in their N-terminal parts. Repeat structures showing variation in number and sequence were present in all strains and may be of relevance to the immunogenicities of the enzymes. Protease activities in cultures of the streptococcal strains were associated with species of different molecular masses ranging from 130 to 200 kDa, suggesting posttranslational processing possibly as a result of autoproteolysis at post-proline peptide bonds in the N-terminal parts of the molecules. Comparison of deduced amino acid sequences revealed a 94% similarity between *S. oralis* and *S. mitis* IgA1 proteases and a 75 to 79% similarity between IgA1 proteases of these species and those of *S. pneumoniae* and *S. sanguis*, respectively. Combined with the results of RFLP analyses using different *iga* gene fragments as probes, the results of nucleotide sequence comparisons provide evidence of horizontal transfer of *iga* gene sequences among individual strains of *S. sanguis* as well as among *S. mitis* and the two species *S. pneumoniae* and *S. oralis*. While *iga* genes of *S. sanguis* and *S. oralis* were highly homogeneous, the genes of *S. pneumoniae* and *S. mitis* showed extensive polymorphism reflected in different degrees of antigenic diversity.

Bacterial immunoglobulin A1 (IgA1) proteases are highly specific endopeptidases that cleave the heavy chain of human IgA1, including its secretory form (S-IgA1), in the hinge region. Such proteases are secreted by a small number of bacteria associated with humans (reviewed in references 20 and 28). IgA1 protease-producing bacteria include the mucosal pathogens *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Ureaplasma urealyticum* as well as some members of resident oral and pharyngeal microfloras. Among the latter are species of *Prevotella* and *Capnocytophaga*, *Streptococcus sanguis*, *Streptococcus oralis*, and *Streptococcus mitis* biovar 1.

Three different classes of proteinases are represented among the IgA1 proteases, illustrating that cleavage of human IgA1 is a property that has evolved among bacteria through convergent evolution following at least three independent lines. Molecular characterizations have revealed that the IgA1 proteases of *Haemophilus* and *Neisseria* are genetically related serine proteinases (1, 25, 29, 30) and that those of *S. sanguis* and *S. pneumoniae* are metalloproteinases (8, 32, 40). Studies using specific inhibitors indicate that the IgA1 protease produced by *Prevotella melaninogenica* is a cysteine proteinase (26). IgA1 proteases are postproline endopeptidases, and those of streptococcal origin cleave one and the same Pro-Thr peptide bond at positions 227 to 228 in the human IgA1 heavy chain (18, 34).

The enzymes have been shown to be active in vivo, and by cleaving human IgA1 they interfere with the protective functions of the principal mediator of specific immunity of the upper respiratory tract (reviewed in reference 20). Consequently, these proteases are thought to be important for the ability of the bacteria to colonize human mucosal surfaces in the presence of specific S-IgA1 antibodies. Furthermore, they may constitute an important factor in the pathogenesis of invasive infections (20). Besides, it has been proposed that increased colonization of the pharynges of infants with IgA1 protease-producing bacteria, in particular *S. mitis* biovar 1, may compromise S-IgA-mediated protection against allergens and lead to atopic sensitization (17).

The *iga* genes encoding IgA1 protease from *S. sanguis* and *S. pneumoniae* have been characterized previously (8, 32, 40). Here we report on the cloning and sequencing of *iga* genes from the other two *Streptococcus* species, *S. oralis* and *S. mitis*, known to produce IgA1-cleaving activity. Furthermore, we have sequenced *iga* genes from an additional seven strains of *S. sanguis* representing the four different biovars of this species.

MATERIALS AND METHODS

Bacterial strains and cloning vectors. The 27 streptococcal strains included in this study, all of which produce IgA1 protease activities, were identified as

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S. sanguis biovar 1 SK1 (ATCC 10556[T]) and SK85; *S. sanguis* biovar 2 SK4 and SK115; *S. sanguis* biovar 3 SK161 and SK162; *S. sanguis* biovar 4 SK49 and SK112; *S. mitis* biovar 1 SK141, SK286, SK564, SK595, SK597, SK599, SK601, SK603, SK605, SK609, and SK610; *S. oralis* SK2 (ATCC 10557), SK23 (NCTC 11427), SK39, SK100, SK105, SK153, and SK155; and *S. pneumoniae* PK81 by using identification principles described previously (19). In addition to the 11 strains of *S. mitis* listed, 44 isolates of that species were included in the study (see Results). The streptococci were grown at 37°C in an atmosphere of air plus 5% CO_2 on blood agar and in Todd-Hewitt broth (Difco, Detroit, Mich.) or, for protein analyses, 2× YT medium (35). The latter medium is devoid of high-molecular-weight proteins. Bacteriophage λL47.1 (22) was used as a *Bam*HI substitution vector, and recombinant phages were plated on *Escherichia coli* K802 (42). *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.) was used as a host for propagation of recombinant forms of the phages M13mp18 and M13mp19 (43) as well as for derivatives of pUC18. *E. coli* XL1-Blue clones were grown in 2× YT medium supplemented with antibiotics when appropriate. DNA fragments generated by PCR amplification were cloned into plasmid pCRII with a TA cloning kit (R&D Systems Europe Ltd., Abingdon, United Kingdom).

Southern blot analyses. Whole-cell DNA was isolated as described previously (12). Approximately 1 μg was digested with *Eco*RI or *Msp*I, treated with RNase, and electrophoresed in a 1% agarose gel, and the DNA fragments were transferred and fixed by UV radiation (UV Stratalinker; Stratagene) onto a Nytran nylon membrane (Schleicher & Schuell, Keene, N.H.). Hybridizations were performed according to the method of Sambrook et al. (35) with modifications as described previously (32). Notably, the low-stringency hybridization protocol included a gradual decline in hybridization temperature which presumably favors specific annealing. Two hybridization probes, each representing half of the *iga* gene of *S. sanguis* strain SK1 (ATCC 10556) (bp 571 to 3395 and 3369 to 5702, respectively, in the published *iga* gene sequence [8]), have been described previously (32). The hybridization probe representing the *S. pneumoniae* PK81 *iga* gene contained bp 1 to 4722 of the published sequence (32) previously cloned into pBluescriptII, and the *S. oralis* SK2 *iga* gene probe consisted of the 6.0-kb insert of plasmid pTB1 isolated in this study. Vector sequences were removed from the DNA probes by digestion with appropriate restriction enzymes followed by agarose gel electrophoresis and elution with a GeneClean kit (Bio 101, Vista, Calif.). The two *S. mitis* SK141 *iga* gene probes were the hybridizing DNA restriction fragments of λSK141*iga*1 described in this study divided into the gene's 5′ and 3′ parts at the *Xba*I site. The DNA fragments used for hybridization probes were labelled with [α-³²P]dATP by nick translation (35).

Construction and screening of genomic libraries. For *S. mitis* SK141 a *Sau*3A partial digest of whole-cell DNA was fractionated by agarose gel electrophoresis. Fragments in the size range 10 to 17 kb were isolated by electroelution and used to construct a genomic library with λL47.1 as a *Bam*HI substitution vector. Recombinant phages packaged in vitro with Gigapack II Packing Extracts (Stratagene) were plated on *E. coli* K802 as described previously (35), and positive plaques were identified by low-stringency hybridization (32). For *S. oralis* SK2 the genomic library was prepared from partial *Mbo*I-digested whole-cell DNA ligated with *Bam*HI-restricted plasmid pUC18 and transformed into competent *E. coli* XL1-Blue. We used *Mbo*I because the genomic DNA was resistant to cleavage with *Sau*3A. Recombinant plasmids carrying genes encoding IgA1 protease activities were identified among the transformants by the agar overlay method as described previously (2). Briefly, plates with the growing transformants were overlaid with agar containing insolubilized human IgA1 and then Fab fragments released by IgA1 protease activity were captured for subsequent detection on nitrocellulose discs coated with anti-light-chain antibody.

PCR. For amplification of genomic DNA sequences, we used *Taq* DNA polymerase (Life Technologies, Roskilde, Denmark) as recommended by the supplier and 19- to 27-mer oligonucleotides (DNA technology, Aarhus, Denmark) as primers. The thermocycling program consisted of denaturation at 94°C for 5 min and 30 cycles of 94°C for 1 min, 55 or 60°C for 1 min, and 72°C for 2 min followed by an extension at 72°C for 6 min. For cloning of PCR products, we used the pCRII vector.

DNA sequencing. As the DNA template for the sequencing reactions we used either single-stranded DNAs prepared from M13 phages or double-stranded DNAs from plasmids, PCR products, or lambda phage. DNAs from M13 phages were prepared as described previously (35), whereas plasmid DNAs were prepared as recommended by the supplier of the sequencing kit. Lambda phage DNA prepared as described previously (35) and PCR products were purified with Wizard Minicolumns (Promega, Madison, Wis.). Individual sequence reactions were done with a *Taq* DyeDeoxy-Terminator cycle sequencing kit (Perkin-Elmer, Foster City, Calif.) and analyzed with an Applied Biosystem DNA sequencer (Perkin-Elmer). As sequencing primers, we used the universal sequencing primers as well as synthetic oligonucleotides (DNA technology) designed on the basis of preceding sequences. The *iga* gene sequences were determined by sequencing of both strands. Computer analysis of the sequences was performed with the program manual for the Wisconsin Package (Genetics Computer Group, Madison, Wis.) and PILEUP for multiple sequence analysis, GAP for calculation of pairwise homologies, and FASTA for database searching.

IgA1 protease assay. Proteins in the supernatant of an overnight culture in 2× YT medium were concentrated 10- to 100-fold by size exclusion centrifugation with Centriprep concentrators (Amicon, Beverly, Mass.) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). IgA1
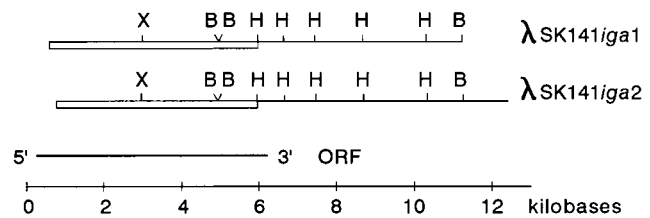

FIG. 1. Structure of the *S. mitis* SK141 *iga* gene. Restriction maps of the two analyzed recombinant lambda phage inserts with homology to the *S. sanguis iga* gene probes are shown. The culture supernatant of *E. coli* K802 infected with λSK141*iga*1 possessed IgA1 cleaving activity. Symbols: H, *Hin*dIII; B, *Bam*HI; X, *Xba*I. The presence of two closely located *Bam*HI sites was revealed by sequence analysis only. Restriction fragments hybridizing to the two *iga* gene probes from *S. sanguis* are boxed. The deduced orientation of the *iga* gene is indicated by 5′ and 3′. The location of the large ORF is indicated below. Note that in the 5′ end of the *iga* gene, this region extends beyond the *S. mitis* SK141 genomic sequences cloned in the lambda phages as described in the text.

protease activity in the gel after electrophoresis was detected as described previously (32). Briefly, inhibition of SDS was overcome and proteins were renatured by incubation of the gel for 1 h in 50 mM Tris-HCl (pH 7.4) containing 0.154 M NaCl, 1% Triton X-100, and 0.5% bovine serum albumin. Subsequently, the gel was laid on a polyvinylidene difluoride membrane onto which human myeloma IgA1 had been immobilized via its Fc part in advance by the following procedure: the membrane was incubated first with rabbit antibody directed against mouse Ig, next with a murine monoclonal antibody recognizing the Fc fragment of human IgA1, and finally with purified human myeloma IgA1 (with κ light chains). Proteins in the gel were allowed to react with IgA1 on the membrane for 2 h at 37°C. After the membrane was washed, cleavage of immobilized IgA1 was visualized by incubation with peroxidase-conjugated rabbit antibody to human κ light chains, followed by incubation with a chromogenic substrate. In this assay, IgA1 protease activity in the gel is reflected on the membrane as a loss of light chains (as part of Fab fragments) and detected on the membrane as a lack of staining.

Nucleotide sequence accession numbers. The nucleotide sequences of the streptococcal *iga* genes reported here have been deposited in the EMBL nucleotide sequence database. The *iga* genes of the listed strains have been assigned the following accession numbers: *S. sanguis* SK4, Y13459; *S. sanguis* SK49, Y13460; *S. sanguis* SK85, Y13461; *S. sanguis* SK112, Y13455; *S. sanguis* SK115, Y13456; *S. sanguis* SK161, Y13457; *S. sanguis* SK162, Y13458; *S. mitis* SK141, Y10285; *S. oralis* SK2, Y10286; and *S. oralis* SK23, Y13224.

RESULTS

Cloning, isolation, and sequencing of streptococcal *iga* genes. We have previously shown by hybridization that the *iga* genes encoding IgA1 protease produced by *S. sanguis*, *S. pneumoniae*, *S. oralis*, and *S. mitis* biovar 1 are all related (32). Therefore, each of two fragments of the *S. sanguis iga* gene was used as a radioactively labelled probe to screen a λL47.1 phage library of whole-cell DNA from *S. mitis* SK141. Two positive recombinant phages, λSK141*iga*1 and λSK141*iga*2, were isolated, and their DNAs were extracted. Sites for the restriction enzymes *Eco*RI, *Hin*dIII, and *Bam*HI were mapped within DNA from each of the recombinant phages. The location and orientation of the *iga* gene were determined by Southern blot analyses of phage DNA restricted with the same enzymes and with the two *S. sanguis iga* gene fragments as probes (Fig. 1). Among the two positive phages, only λSK141*iga*1 was found to encode IgA1-cleaving activity as detected in the *E. coli* K802 culture lysate. Overlapping restriction fragments of the hybridizing 5.5-kb part of this phage were subcloned into phages M13mp18 and M13mp19 and sequenced. Comparison with the published *iga* gene sequences from *S. sanguis* and *S. pneumoniae* (8, 32, 40) suggested that the resulting sequence lacked 113 bp in the 5′ ends, which was not contained within λSK141*iga*1, and at least 46 bp in the 3′ ends of the genes. Since λSK141*iga*1 lacked the 5′ part of the *iga* gene, the observed expression of IgA1 protease activity was presumably driven by an in-frame promoter in the λL47.1 vector. Alterna-
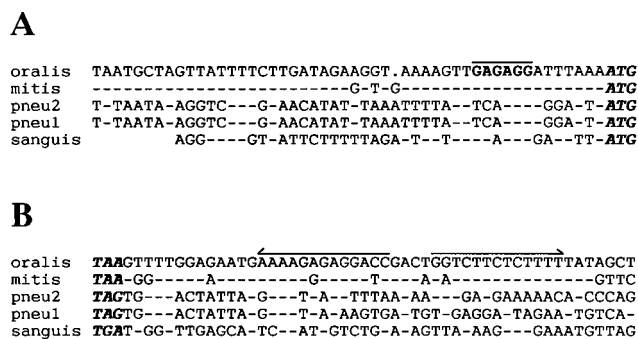
A

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oralis   TAATGCTAGTTATTTTCTTGATAGAAGGT.AAAAGTTGAGAGGATTTAAAATG
mitis    -------------------------G-T-G---------------------ATG
pneu2    T-TAATA-AGGTC---G-AACATAT-TAAATTTTA--TCA----GGA-T-ATG
pneu1    T-TAATA-AGGTC---G-AACATAT-TAAATTTTA--TCA----GGA-T-ATG
sanguis      AGG----GT-ATTCTTTTTAGA-T--T----A---GA--TT-ATG
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B

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oralis   TAAGTTTTGGAGAATGAAAAGAGAGGACCGACTGGTCTTCTCTTTTTATAGCT
mitis    TAA-GG-----A---------G-----T----A-A--------------GTTC
pneu2    TAGTG---ACTATTA-G---T-A--TTTAA-AA---GA-GAAAAACA-CCCAG
pneu1    TAGTG---ACTATTA-G---T-A-AAGTGA-TGT-GAGGA-TAGAA-TGTCA-
sanguis  TGAT-GG-TTGAGCA-TC--AT-GTCTG-A-AGTTA-AAG---GAAATGTTAG
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FIG. 2. Comparison of sequences upstream and downstream of different streptococcal *iga* genes. Abbreviations: oralis, *S. oralis* SK2 (this study); mitis, *S. mitis* SK141 (this study); pneu2, *S. pneumoniae* P110 (40); pneu1, *S. pneumoniae* PK81 (32); sanguis, *S. sanguis* ATCC 10556 (8). Dashes indicate nucleotides identical to nucleotides of the *S. oralis* sequence. (A) Upstream sequences. The ATG initiation codons are indicated by boldface italic type. The putative ribosome binding site in the *S. oralis* and *S. mitis* sequences is overlined. The dot in the *S. oralis* sequence marks a gap introduced for the alignment. (B) Downstream sequences. The stop codons of the respective *iga* genes are indicated by boldface italic type. The putative transcription terminators in the *S. oralis* and *S. mitis* sequences are indicated by divergent arrows.

tively, a sequence within the *iga* gene incidentally resembled a gram-negative gene promoter and was recognized as such by *E. coli* as previously described for the *S. sanguis iga* gene (8). The sequence of the 5′ end of the *S. mitis* SK141 *iga* gene was obtained by direct sequencing of a PCR fragment generated from SK141 genomic DNA by a primer based on the sequences from λSK141*iga*1 combined with one based on sequences upstream of the *S. oralis* SK2 *iga* gene determined in parallel (see below). The 3′-end sequence of the *S. mitis* SK141 *iga* gene was completed by direct sequencing of λSK141*iga*1 DNA with a primer based on the established sequence.

An unsuccessful attempt was made to amplify by PCR the *iga* genes from an additional six strains of *S. mitis* with several different combinations of the oligonucleotides synthesized for sequencing of the *S. mitis* SK141 and *S. oralis* SK2 *iga* genes. This result suggests that *iga* genes are highly heterogeneous among strains of *S. mitis.*

The *S. oralis* SK2 (ATCC 10557) *iga* gene was isolated from a library of whole-cell DNA cloned into plasmid pUC18 and transformed into *E. coli* XL1-Blue. Recombinant plasmids encoding IgA1 protease activity were identified among the transformants by the agar overlay method. A single positive clone, pTB1, containing an insert of 6.0 kb was selected for further analyses. The nucleotide sequence of the pTB1 insert was determined and revealed that this plasmid contained the complete *iga* gene from *S. oralis* SK2. The observed expression of IgA1 protease activity from the *S. oralis iga* gene in *E. coli* host cells harboring plasmid pTB1 was not examined further. It is not likely that the streptococcal *iga* gene promoter is recognized in gram-negative bacteria. Presumably, a promoter different from the authentic one functioned in *E. coli*, as was observed for the *S. sanguis iga* gene (8).

Both the *S. oralis* SK2 and the *S. mitis* SK141 *iga* gene sequences revealed a large open reading frame (ORF) starting with an ATG triplet and having the potential of encoding polypeptides of 1,839 and 1,854 amino acids, respectively, with extensive homology to the other known streptococcal IgA1 proteases (see below). In the 50-bp region preceding the ORF, these two novel *iga* gene sequences were 95% identical whereas they had no significant homology with the corresponding sequences from *S. sanguis* and *S. pneumoniae* (Fig. 2A). All the

streptococcal *iga* genes analyzed contained a plausible ribosome binding site just upstream of the initiation codon, whereas a conserved −10 promoter sequence element was not evident (Fig. 2A). The authentic transcription start site of the *iga* genes has not been determined (8). In the nucleotide sequences downstream of the ORF, the *S. oralis* and *S. mitis* strains analyzed also displayed homologies not found among *S. sanguis* and *S. pneumoniae* strains (Fig. 2B). This region included an inverted repeat structure which may constitute a transcription terminator.

The nucleotide sequence of the *iga* gene from an additional strain of *S. oralis*, SK23 (NTCT 11427), was obtained by direct sequencing of overlapping DNA fragments generated by PCR amplification of whole-cell DNA with, as primers, different combinations of the oligonucleotides synthesized for sequencing of the two *iga* genes described above.

The *iga* gene sequence from *S. sanguis* ATCC 10556 (SK1) has been published previously (8). To evaluate the diversity of the *iga* gene in *S. sanguis*, we sequenced the genes from an additional seven strains of the species. Together with SK1, these strains represented each of the four biovars of *S. sanguis* by two strains. For the sequencing, we amplified by PCR each of the seven *iga* genes in three DNA fragments corresponding to bp 1 to 716, 571 to 3395, and 3369 to 5702 in the published *iga* gene sequence of ATCC 10556 (8). These three overlapping fragments cover the ORF of the *iga* gene. For each strain, the DNA fragment corresponding to bp 1 to 716 was sequenced directly with the PCR product as the template in the sequencing reactions, whereas the other two DNA fragments were sequenced after cloning into plasmid pCRII and with DNA from the resulting recombinant plasmids as the template. A few errors in the sequences of the last two fragments might have occurred during the PCR if the *Taq* DNA polymerase made mistakes that were copied in the cloning procedure. Three single-base differences were detected when fragments containing a total of 5 kb were resequenced directly from PCR-amplified templates. Thus, the expected frequency of PCR-generated errors in the sequences was less than 1 in 1,000.

Homologies among streptococcal IgA1 proteases. Together with the three streptococcal *iga* genes published previously (8, 32, 40), the results from this study enabled us to deduce and compare amino acid sequences of the IgA1 proteases from eight strains of *S. sanguis*, one strain of *S. mitis*, two strains of *S. oralis*, and two strains of *S. pneumoniae*. The resulting alignment is shown in Fig. 3A. These 13 streptococcal IgA1 proteases were highly homologous except for a region in their N-terminal thirds which differed in both length and sequence. None of the streptococcal *iga* genes showed significant homology to the serine-type IgA1 proteases from *Neisseria* and *Haemophilus* or to other sequences in the GenBank and EMBL databases.

All the streptococcal IgA1 proteases contained the sequence HEMTH (at amino acid positions 1495 to 1499 in the *S. sanguis* SK1 IgA1 protease sequence) followed by an E residue 20 amino acids downstream (Fig. 3). These features are characteristic of the active sites of bacterial metalloproteinases (38), and site-directed mutagenesis of this motif in the *S. sanguis* SK1 IgA1 protease has shown that it is essential for enzyme activity (8). Thus, all the streptococcal IgA1 proteases share this catalytic mechanism. Based on its exclusive use in other bacterial metalloproteinases, Zn is the most likely ligand.

At the N terminus, a peptide of 42 amino acids with typical features of a signal sequence (39) was present in all the streptococcal IgA1 proteases analyzed. Taking this signal peptide into account, the molecular masses of the deduced proteases

A

↓ signal peptidase site   ♦♦♦♦ cell wall anchor

FIG. 3. Alignment of streptococcal IgA1 protease sequences.

Zn-binding *****   *

sang1, sang161, sang112, sang49, sang4, sang115, sang85, sang162, mitis141, oralis2, oralis23, pneu1, pneu2

B

Variable region

LPNTG repeats   HEMTH.....E

FIG. 3. Comparison of streptococcal IgA1 protease sequences. The amino acid sequences were deduced from the *iga* gene sequences of *S. sanguis* ATCC 10556 from reference 8 (sang1), *S. sanguis* SK161, SK112, SK49, SK4, SK115, SK85, and SK162 from this study (sang161, sang112, sang49, sang4, sang115, sang85, and sang162, respectively), *S. mitis* SK141 from this study (mitis141), *S. oralis* SK2 and SK23 from this study (oralis2 and oralis23), *S. pneumoniae* PK81 from reference 32 (pneu1), and *S. pneumoniae* P110 from reference 40 (pneu2). (A) Alignment of the 13 IgA1 protease sequences. Gaps, indicated by dots, were introduced by the PILEUP program. Identical amino acids are in white letters on a black background. The proposed signal peptidase cleavage site, the cell wall anchor motif, and the putative zinc-binding sequence are indicated. (B) Schematic, consensus structure of the streptococcal IgA1 proteases. Black boxes indicate hydrophobic regions proposed to serve as transmembrane domains in the signal peptide and in combination with the cell wall anchor motif.
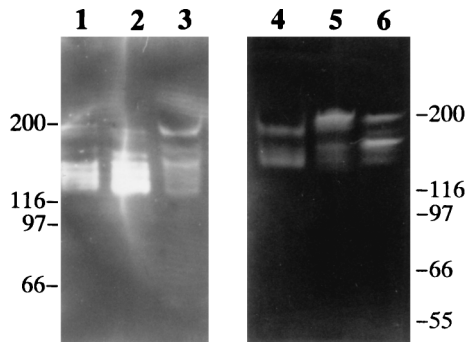
185

FIG. 4. Examples of IgA1 protease profiles of different streptococcal species. Proteins in culture supernatants concentrated 10- to 100-fold were separated according to size by SDS-PAGE, renatured in the gel, and allowed to react for 2 h with human IgA1 bound via its Fc part to a filter. IgA1 cleaving activity diffusing out of the gel was visualized on the filter by lack of reaction with anti-light-chain antibodies (bands of reduced or no staining). Lane 1, *S. sanguis* SK4; lane 2, *S. sanguis* SK115; lane 3, *S. sanguis* SK162; lane 4, *S. oralis* SK2; lane 5, *S. mitis* SK141, lane 6, *S. pneumoniae* PK81. Molecular mass markers in kilodaltons are indicated for the two gels.

were approximately 200,000 Da. As with the IgA1 protease activity secreted by *S. pneumoniae* (32), IgA1-cleaving activities in culture supernatants of strains representing *S. sanguis*, *S. mitis*, and *S. oralis* could be ascribed to several molecular species with apparent molecular masses ranging from 130 to 200 kDa (Fig. 4). For the *S. sanguis* and *S. mitis* strains tested, electrophoretically determined M_rs of the high-molecular-weight form were in agreement with the calculated molecular masses, whereas for *S. oralis* SK2, the largest protein detected with IgA1 cleaving activity was below 200 kDa. Upon prolonged storage, conversion of the larger molecular forms of active IgA1 protease into the smaller forms was observed (data not shown).

At amino acid positions 96 to 100, all 13 streptococcal IgA1 proteases analyzed contained a typical gram-positive cell wall anchor motif, i.e., the sequence LPNTG followed by a hydrophobic stretch (positions 103 to 125) with the potential of spanning the cytoplasmic membrane and a charged region rich in lysines (Fig. 3) (6, 15). As noted previously (8, 40), the charged lysine-rich region was followed by another hydrophobic region (positions 133 to 162) (Fig. 3).

In the N-terminal third, the IgA1 proteases were quite different. In this region (corresponding to amino acid positions 350 to 546 in the *S. sanguis* SK1 sequence) all the proteases contained repeat structures. *S. sanguis* strains SK161, SK112, SK49, SK4, SK115, SK85, and SK162; *S. mitis* strain SK141; and *S. oralis* strains SK2 and SK23 contained 10, 10, 10, 11, 10, 11, 13, 10, 9, and 9 tandem repeats, respectively, of a sequence motif similar to the 20-amino-acid repeat sequence represented by 10 copies in the *S. sanguis* SK1 IgA1 protease (8). The two pneumococcal IgA1 protease sequences contained three tandemly arranged copies of a different sequence of 17 amino acids in addition to several short duplicated sequence elements with homology to the repeat structures found in the other streptococcal IgA1 proteases (32, 40). Another notable feature of the N-terminal thirds of the IgA1 proteases was the high proportion of proline residues.

At the C terminus, the IgA1 proteases analyzed were very similar except for that of *S. sanguis* SK1, which differed markedly. This difference is caused by deletion of a single nucleotide in the SK1 *iga* gene sequence (at position 5538 in the published sequence [8]), resulting in a frameshift mutation. We con-

firmed this difference by sequencing the SK1 *iga* gene in this region.

The degree of nucleotide sequence homology among *iga* genes representing the four different streptococcal species varied from 65 to 93% identity. Correspondingly, the amino acid sequence similarity, i.e., allowing for differences, including functionally conserved amino acids, among the deduced IgA1 protease sequences varied from 69 to 94% (Fig. 5). The highest degree of interspecies identity (92 to 93%) was displayed by the *iga* genes from *S. mitis* SK141 and the two strains of *S. oralis* analyzed. In contrast, the *iga* genes from strains of *S. sanguis*, which were very homogeneous within the species (92 to 100% identity), were more distantly related to those of the other species (65 to 70% identity). The two *S. pneumoniae iga* genes were 88% identical and showed homologies to the *iga* genes of the other species ranging from 65 to 79%. In the region upstream of the *iga* gene the two published pneumococcal sequences were almost identical. This identity extended 1.3 kb into the *iga* gene except for two nucleotides. The remaining part of the *S. pneumoniae iga* gene was highly heterogeneous. This difference in homology within the gene presumably reflects a recent recombinational event.

Restriction fragment length polymorphism analyses. To compare *iga* genes among *S. oralis* and *S. mitis* strains, we performed Southern blot analyses on *Msp*I-digested whole-cell DNA from 6 strains of *S. oralis* and 11 strains of *S. mitis* using as probes each of the *iga* genes from *S. oralis* SK2 and *S. pneumoniae* PK81 under stringent hybridization conditions. For each of the *S. oralis* strains analyzed, a unique pattern formed when *Msp*I restriction fragments hybridized strongly with the *S. oralis* SK2 *iga* gene probe. At least one fragment from each strain hybridized also with the *S. pneumoniae* PK81 probe (data not shown). The 11 strains of *S. mitis* showed an extremely diverse pattern of *Msp*I fragments recognized by the two probes. Five of the strains hybridized with the *S. oralis* SK2 *iga* gene probe, and nine hybridized with the *S. pneumoniae* PK81 probe, whereas two strains hybridized with neither of the probes (Fig. 6). In the genomes of some of the hybridizing strains (SK141, SK286, SK610, and SK595), there were *Msp*I fragments that were recognized by the *S. oralis* SK2 probe only, whereas in other genomes (SK564, SK599, SK597, SK601, and

strain	Nucleotide sequence homology between *iga* genes (percent identity)												
	sang 1	sang 161	sang 112	sang 49	sang 4	sang 115	sang 85	sang 162	mitis 141	oralis 2	oralis 23	pneu 1	pneu 2
sang 1		100	93	93	93	93	93	94	68	68	69	67	67
sang 161	99		93	93	93	93	93	93	68	69	69	67	67
sang 112	94	95		99	93	93	93	93	68	69	69	66	67
sang 49	94	95	100		93	93	93	93	68	69	70	67	67
sang 4	95	95	95	95		93	92	92	68	69	69	66	66
sang 115	94	95	95	95	95		93	92	68	69	69	66	66
sang 85	95	95	95	95	94	95		93	69	69	70	67	67
sang 162	96	96	95	95	95	95	95		69	69	69	67	65
mitis 141	75	75	75	75	76	75	75	76		93	92	73	77
oralis 2	75	76	75	75	76	76	75	76	95		92	71	76
oralis 23	75	76	75	75	76	76	75	76	94	94		74	79
pneu 1	69	70	69	69	69	69	70	70	76	76	76		88
pneu 2	69	70	70	70	69	70	69	69	79	79	79	89	

Amino acid sequence homology between IgA1 proteases (percent similarity)

FIG. 5. Percent homologies between different streptococcal *iga* genes and between the deduced IgA1 proteases. Species and strain designations are described in the legend to Fig. 3. One hundred percent homology means ≥99.5% homology. Abbreviations are as defined in the legend to Fig. 3.
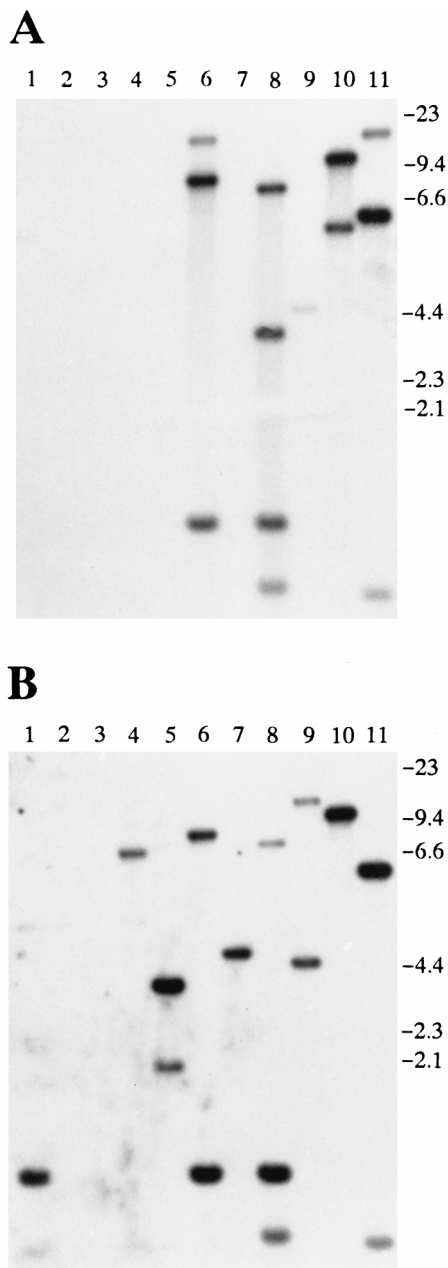
FIG. 6. Southern blot analysis of *S. mitis* biovar 1 strains. Whole-cell DNA was restricted with *Msp*I, electrophoresed in an agarose gel, transferred to a nylon membrane, and hybridized with the *iga* gene probe from *S. oralis* SK2 (A) or *S. pneumoniae* PK81 (B). Lane 1, SK609; lane 2, SK605; lane 3, SK603; lane 4, SK601; lane 5, SK597; lane 6, SK595; lane 7, SK599; lane 8, SK610; lane 9, SK564; lane 10, SK286; lane 11, SK141. Molecular size markers (in kilobases) are indicated to the right.

SK609), there were fragments hybridizing exclusively with the *S. pneumoniae* PK81 *iga* probe.

The high degree of diversity of the *iga* genes observed in *S. mitis* strains was confirmed by *Eco*RI restriction fragment length polymorphism typing with two *iga* gene probes from *S. mitis* SK141 representing the 5′ and 3′ parts of the gene. A total of 48 strains of *S. mitis* biovar 1, of which 26 showed IgA1 protease activities, and seven strains of *S. mitis* biovar 2, all devoid of IgA1 protease activity, were included. Except for one

strain (SK272, biovar 1), only strains producing detectable IgA1 protease activities showed hybridization with the two probes. Of the 26 IgA1 protease-positive strains, 12 hybridized with both the 5′ and 3′ parts of the SK141 *iga* gene, 6 hybridized with the 3′ part only, and 8 were not recognized by either of the two probes. All hybridizing strains showed a distinct pattern of *Eco*RI fragments recognized by the two probes (data not shown).

DISCUSSION

Results from this study together with published *iga* gene sequences (8, 32, 40) allowed us to deduce and compare the amino acid sequences of IgA1 proteases from 13 streptococcal strains representing the four species of the genus known to possess IgA1 cleaving activity, i.e., *S. sanguis*, *S. oralis*, *S. mitis*, and *S. pneumoniae*. The IgA1 proteases of the strains were found to be homologous enzymes, and the comparison revealed conserved as well as variable areas. Among the conserved areas, all the IgA1 proteases contained a zinc-binding consensus sequence, indicating that they are Zn metalloproteinases.

In gram-positive bacteria many surface proteins are anchored to the cell wall via a structure close to the C terminus consisting of an LPXTG sequence motif followed by a membrane-spanning hydrophobic region and a charged sequence (6, 15, 27). The amino acid sequence comparison confirmed the observation previously made by Wani et al. (40) that in the streptococcal IgA1 proteases such a structure is found in the N-terminal part. Its exceptional location implies that it cannot function in the traditional way as a cell wall anchor for surface exposure. However, in the IgA1 proteases the anchor structure is followed by another hydrophobic region and a large area rich in prolines (Fig. 3). For surface-exposed proteins, a sequence containing a high proportion of proline residues is commonly associated with the cell wall (15). Provided that the cell wall anchor motif combined with two membrane-spanning regions followed by a wall-associated region rich in prolines are functional, one may speculate that the protease molecule is coupled to the cell wall via its N terminus, traverses the cytoplasmic membrane twice, and spans the cell wall, ending up on the surface of the bacterium, from where it is released by proteolysis.

Another characteristic common to the streptococcal IgA1 proteases was tandemly repeated sequence elements in their N-terminal parts. Repeat structures are found in most surface-exposed and secreted proteins of streptococci and may provide a means for variation through intragenic homologous recombination (15). Variation in number and sequence of repeats may contribute to antigenic diversity and, hence, to immune evasion (15). However, studying the *S. sanguis* IgA1 protease, Gilbert and coworkers (9) demonstrated that the repeat region is not essential for enzyme activity. This finding is of interest, since by SDS-PAGE we found that active IgA1 proteases in streptococcal culture supernatants varied in M_r from ~200,000 (~200K) to 130K, 200K being the M_r calculated for the entire translation product. Although nonenzymatic hydrolysis of Asp-Pro bonds has been observed after extensive boiling of certain streptococcal proteins in acidic sample buffer (21, 41), this phenomenon is not a relevant explanation in the present case, because the preparation did not involve acidic conditions and, moreover, the location of Asp-Pro peptide bonds in the IgA1 proteases, in *S. sanguis* in particular, could not account for the observed sizes. Thus, the multiple molecular forms indicate posttranslational, possibly postsecretional, processing of the proteases.

Although the sites cleaved during processing have not been identified, the N-terminal part is likely to be involved. The smallest molecular version of the active protease results from removal of portion(s) corresponding to ~70 kDa from the primary translation product. If such processing involved the C-terminal part only, it would deprive the enzyme of its active site (Fig. 3). As previously suggested (32), processing at the N terminus might involve autoproteolysis, as occurs in the processing of the serine-type IgA1 proteases produced by *N. gonorrhoeae*, *N. meningitidis*, and *H. influenzae* (25, 29, 30). Autoproteolytic cleavage of the latter enzymes is known to involve also post-proline bonds different from the Pro-Ser or Pro-Thr peptide bond cleaved in human IgA1 (20). By analogy, autoproteolysis of streptococcal IgA1 proteases, if it occurs, might involve not only Pro-Thr, as in the cleavage of the human α1 chain, but also Pro-Ser and other post-proline bonds, which are abundant in the N-terminal thirds of the streptococcal enzymes (Fig. 3A).

Gilbert and coworkers also found that the repeat region of the *S. sanguis* protease was immunogenic when it was injected in recombinant form into rabbits and that antibodies reacting with this region of the protease were present in normal human saliva in the form of S-IgA (9). Notably, such antibodies did not inhibit enzyme activity (9). Based on these observations and the overall structural similarity of the streptococcal IgA1 proteases, epitopes located C-terminally to the repeat region are likely to be the targets of neutralizing antibodies to these enzymes. Cross-inhibition experiments with such antibodies, obtained from rabbits immunized with concentrated culture supernatants or from individuals recovering from streptococcal disease (34), have revealed considerable serological diversity of IgA1 proteases in *S. pneumoniae* (23) and *S. mitis* (32a). In contrast, very limited diversity was found in *S. sanguis* and *S. oralis* (34). These results are in agreement with the structural diversity of IgA1 protease genes in each of the species. Thus, nucleotide sequence comparison combined with Southern blot analysis revealed that in *S. sanguis* and *S. oralis* the *iga* gene is relatively conserved whereas in *S. mitis* it is extremely heterogeneous. In *S. pneumoniae* strains, we have previously shown that *iga* genes are diverse (32), though not to the degree observed in *S. mitis* strains.

In a previous study of IgA1 protease-inhibiting antibodies (33), sera and saliva of most healthy subjects were found to be devoid of activities inhibiting the protease of *S. sanguis*, which is a lifelong member of the oral flora. This finding suggests that the human host responds rarely, if at all, to the activity-relevant structures located C-terminally to the repeat region of the *S. sanguis* protease. Yet, as mentioned above, antibodies to the repeat region are present in saliva (9). Comparable immunological data have been obtained for other microbial, including streptococcal, proteins containing repeat regions identified as immunodominant (7, 37). For these proteins it is believed that an exaggerated response to the immunodominant repeats suppresses responses to other epitopes (7, 37), as with the immunodominance demonstrated for certain epitopes in other proteins (3, 4). Assuming, however, that N-terminal processing, as observed in vitro, occurs also during colonization of the human host, it remains to be explained why the enzymatically active part of the *S. sanguis* protease molecule devoid of the repeat structures rarely induces neutralizing secretory and systemic antibodies.

Contrary to the results for the *S. sanguis* IgA1 protease, sera and saliva of healthy humans contain neutralizing antibodies to the IgA1 proteases of *S. oralis* and *S. mitis* (33). The immunogenicities of distinct regions of these proteases have not been examined. However, the different result for *S. sanguis* com-

A

B
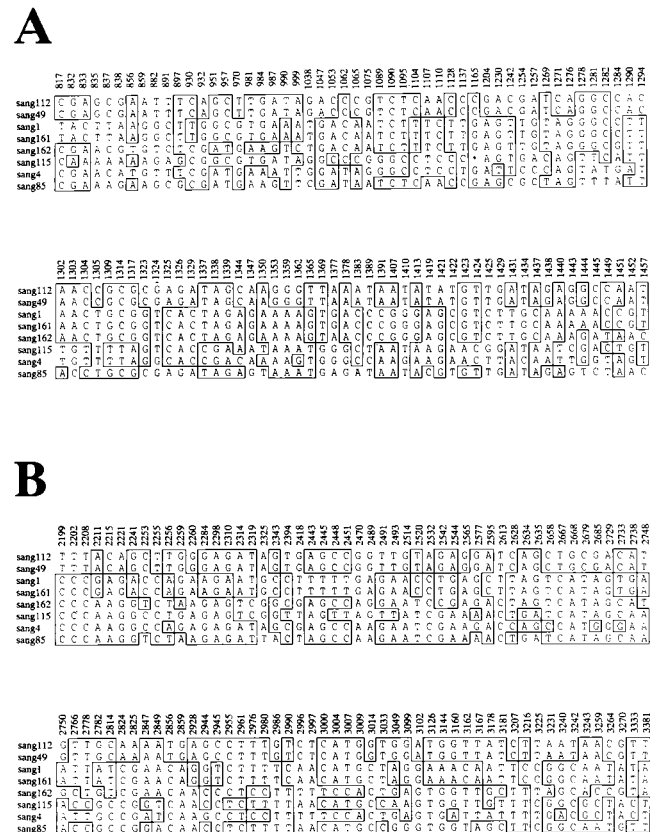
FIG. 7. Informative polymorphic nucleotide sites in the eight *S. sanguis iga* genes in the regions corresponding to nucleotides 817 to 1457 and 2199 to 3381 in the published *S. sanguis* ATCC 10556 *iga* gene sequence (8). Each site is numbered according to the ATCC 10556 *iga* sequence. Abbreviations are as defined in the legend to Fig. 3.

pared to results for *S. oralis* and *S. mitis* is remarkable, considering that the three species are closely related and have a common, commensal relationship with the host. In view of the closer relationship of the proteases of *S. oralis* and *S. mitis* than that of the proteases of *S. sanguis* and *S. pneumoniae* (discussed below), we speculate that neutralizing antibodies to the proteases of *S. oralis* and *S. mitis* may be raised by cross-reactive IgA1 proteases of *S. pneumoniae* clones consecutively colonizing humans during childhood and adolescence (10). The results of our previous (24) and ongoing studies of the serological relationship between streptococcal IgA1 proteases corroborate this hypothesis.

Species within *Streptococcus* are naturally competent, and evidence for horizontal DNA transfer within as well as between species has been reported (5, 13, 16, 23, 36). Our results on hybridization between restriction fragments of *iga* genes from the different species strongly indicate that *iga* gene sequences have been transferred between *S. mitis* and two other species, *S. oralis* and *S. pneumoniae*. In some strains of *S. mitis* we found genomic DNA restriction fragments that were highly homologous to the *iga* gene of *S. oralis* SK2 and different from that of *S. pneumoniae* PK81; in other strains we found fragments that were homologous to the *iga* gene of *S. pneumoniae* PK81 and different from that of *S. oralis* SK2, whereas in still others the *iga* gene did not hybridize with that of either of the strains representing the two other streptococcal species. Thus, in *S. mitis* the IgA1 protease gene is a complex mosaic of

sequences closely related to each of the two other species, *S. oralis* and *S. pneumoniae*, and sequences only distantly related to these. Such a mosaic structure is most adequately explained by a process of horizontal transfer in vivo between *iga* genes of the different species.

In *S. sanguis* strains *iga* genes were very homogeneous, with only relatively low homology to the other streptococcal *iga* genes sequenced. A comparison of the eight *S. sanguis iga* gene sequences provides information on the evolutionary origin of the limited variation within the species. The homologies reveal a mosaic-like organization strongly indicative of horizontal genetic exchange between *iga* genes of different clones of *S. sanguis*. Examples of two regions depicting such an organization are shown in Fig. 7, in which the sequence comparison has been condensed to include only informative polymorphic sites, i.e., sites with at least two alleles, each represented by more than one strain. Remnants of recombination between *iga* gene sequences of individual *S. sanguis* strains are illustrated by the patched organization of the homologies to strain SK162: at the informative sites from bp 1038 to 1443, strain SK162 is identical to strains SK1 and SK161, whereas from bp 2945 to 3144, strains SK162 and SK4 are identical. A similar mosaic-like organization has been previously found among the *iga* genes encoding serine-type IgA1 proteases in *N. gonorrhoeae*, *H. influenzae*, and *N. meningitidis* (11, 25, 31). Lack of hybridization between some of the streptococcal *iga* genes, e.g., between those of *S. sanguis* and those of the other three species (32), may constitute a barrier for horizontal transfer of *iga* gene sequences.

The overall relationships among the IgA1 protease gene sequences from the different streptococcal species included in this study are in accordance with the phylogeny of the genus previously suggested on the basis of 16S rRNA sequences (14). However, in our analysis three of the species were represented by only one or two strains. Together with the high degree of intraspecies diversity of the gene, in *S. mitis* in particular, this implies that the sequence comparison presented here does not necessarily reveal in full the phylogenetic relationships of IgA1 proteases among streptococci.

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