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Molecular Characterization of a Major Serotype M49 Group A Streptococcal DNase Gene (*sdaD*)

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Group A streptococci (GAS) express up to four types of secreted DNases. Although GAS infections are correlated with the production of anti-DNase B antibodies, the roles of DNases in the pathogenesis of GAS infections remain unclear. From a λ library of serotype M49 strain CS101 GAS genome, a 2,147-bp fragment expressing DNase activity on an indicator agar was identified and sequenced. One 1,155-bp open reading frame (ORF) was identified in this fragment. This ORF was found to be 48% identical on the amino acid level to group C streptococcal DNase (Sdc). The regions of highest homology corresponded to amino acid residues that were also identified as part of the active site in staphylococcal nuclease. Transcription analysis revealed a specific 1.3-kb mRNA, which corresponded to the size predicted by the promoter and transcription termination signature sequences and indicated a monocistronic mode of transcription. Allelic replacement of the ORF rendered a M49 mutant devoid of extracellular DNase activity when cultured on indicator agar. Virulence parameters such as resistance to phagocytosis were not affected by the mutation. The *sda* gene was cloned and expressed in *Escherichia coli* as a thioredoxin fusion. By performing Ouchterlony immunodiffusion on the recombinant protein and by using protein preparations from culture supernatants of wild-type bacteria and the DNase mutant, the results of immunoreactivity with DNase type-specific polyclonal rabbit antisera classified the DNase as a type D enzyme. Fifty percent of patients with sera exhibiting high titers of antistreptolysin or anti-DNase B antibodies also had SdaD-reactive antibodies in comparison with <10% of serologically normal controls. While the value of recombinant SdaD for diagnostic purposes needs to be clarified, the isogenic DNase mutant pair of M49 should allow the significance of GAS DNase D as a bacterial virulence factor to be determined.

Bacteria of the species *Streptococcus pyogenes* (group A streptococci [GAS]) are important human pathogens. Their pathogenicity is based on the production of numerous virulence factors. Some of these virulence factors are expressed only on the bacterial surface (reviewed by Kehoe [21]) and are under the control of the Mga regulator (27, 28, 34). Other virulence factors are secreted into the bacterial environment. These factors comprise enzymes which act as spreading factors, like streptokinase (24, 25) and hyaluronidase (18), cytolytic streptolysins O and S (22), superantigens like the erythrogenic toxins SpeA (48) and SpeC (14), and a cysteine protease (5).

Extracellular streptococcal DNases have also been implicated as virulence factors (11) because of several features. First, their activity is directed to DNA, a macromolecule crucial for function of any potential target cell. Second, extracellular DNases are produced by each GAS strain tested so far (10). Third, production of nucleases in less-virulent group B, C, and G streptococci is less common, and less enzyme is synthesized than by GAS (10, 30, 38). Fourth, production of anti-DNase antibodies is a common and reliable feature during GAS infections (2). Fifth, nucleases have been implicated as virulence factors in other gram-positive cocci such as *Staphylococcus aureus* (6). However, a specific role in pathogenicity has never been directly shown for GAS DNases.

Attempts to correlate DNase production rates and antibody titers in infected persons to the virulence of specific GAS isolates have not yet been successful (10, 19). Only some anatomical sites of infection, such as in pyoderma, were found to be associated with higher anti-DNase antibody titers (2, 9, 42). Thus, the status of GAS DNases as virulence factors is debatable, and these enzymes could have only an indirect effect on virulence by providing a given GAS strain with oligonucleotides as a source of energy (49).

Since 1958, four different types of GAS DNases have been reported (45, 46). These types were designated DNase A through D and were differentiated by several serological, immunological, and functional parameters (15, 47) such as substrate specificity (DNA only or DNA and RNA), pH optimum, thermal stability, and susceptibility to an inhibitory activity of bacterial tRNA. Although analyzed only by nondenaturing methods, the masses of the four DNases were estimated to be between 25 and 30 kDa. Patient sera as well as rabbit antisera raised against partially purified DNases were found either to inhibit DNase activity in a type-specific manner or to exhibit very little cross-reactivity.

When measuring the type-specific DNase production of more than 100 GAS strains, DNase B was found to be secreted by all isolates tested and to represent a major share of total DNase activity of most strains. DNase C normally contributed almost as much as DNase B to total DNase activity but was expressed by only 50% of the strains. DNases A and D were secreted by about 25 to 30% of the isolates tested (31, 44, 46). The production of DNase B by virtually all GAS strains and the frequent specific antibody response of GAS-infected humans to this antigen prompted investigators to use measurements of anti-DNase B antibody titers as a standard technique to screen for GAS infections (13, 16, 20, 32).

While *S. aureus* extracellular nuclease is one of the most thoroughly studied proteins of these bacteria (e.g., see refer-

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ences 3, 29, 40), GAS DNases still await molecular characterization. Among streptococci, only a group C streptococcal DNase gene (*sdc*) (50) has been sequenced and expressed in foreign host bacteria. In the present investigation, we characterized the major DNase of serotype M49 GAS. These data and the isogenic mutants we generated in the course of this study should help to elucidate the status of GAS DNases as virulence factors of these bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. pyogenes* serotype M49 strain CS101 was provided by P. Cleary, Minneapolis, Minn. *Escherichia coli* DH5α (Gibco BRL, Eggenstein, Germany) served as the gram-negative host for recombinant pFW3 plasmids. *E. coli* BlueMRF and XLOLR (Stratagene, Heidelberg, Germany) were used as hosts for phages Lambda ZAP Express and ExAssist, respectively. *E. coli* GI724 and GI698 (Invitrogen, Leek, The Netherlands) served as hosts for pTrxFus plasmids. The GAS isolates were grown in tryptone soy (CASO) broth (Unipath, Wesel, Germany) or on Todd-Hewitt agar (TH agar; Unipath). For growth of mutant GAS strains, the culture media were supplemented with 60 mg of spectinomycin per liter. All GAS cultures were incubated at 37°C in a 5% CO_2–20% O_2 atmosphere.

E. coli DH5α strains were grown on disk sensitivity testing (DST) agar (Unipath) supplemented with 100 mg of spectinomycin per liter. *E. coli* BlueMRF infected with recombinant λ phages was grown in NZY agar, and *E. coli* XLOLR strains transfected with recombinant ExAssist phagemids were grown on Luria-Bertani (LB) medium supplemented with 50 mg of kanamycin per liter, both according to instructions of the supplier (Stratagene). *E. coli* GI724 and GI698 were propagated in RM medium or on RMG agar, both supplemented with 100 mg of ampicillin per liter, as recommended by the manufacturer (Invitrogen). The *E. coli* isolates were grown at 37°C in ambient air except for strains GI724 and GI698, which were cultured at 30°C.

For visualization of DNase activity, the culture media were supplemented with 500 mg of calf thymus DNA per liter and 250 mg of methyl green per liter (23), and the pH of the medium was adjusted to 7.5.

Phage vectors. *E. coli* phages Lambda ZAP Express (*Bam*HI arms, calf intestinal phosphatase treated) and ExAssist were purchased from Stratagene and used according to the instructions of the manufacturer.

Plasmids. Plasmid pFW3, derived from pSF152 (43), carries a pUC19 origin of replication, an *aad*9 spectinomycin resistance gene, and two multiple cloning sites (37). pFW3 was used for allelic replacement mutagenesis. Recombinant pBK plasmids resulted from ExAssist phagemid transfections and were used according to the supplier's instructions (Stratagene). Plasmid pTrxFus (Invitrogen) contains a ColE1 origin of replication, a TEM-1 β-lactamase gene, and an *E. coli* thioredoxin gene (*trxA*) with a C-terminal multiple cloning site. The *trxA* gene is transcribed from a phage λ p_L promoter which is normally repressed by a λ *c*I repressor. In *E. coli* GI724 and GI698, this repressor is chromosomally encoded within the tryptophan synthesis operon. External supplementation of tryptophan shuts down transcription of the tryptophan synthesis operon and the integrated *c*I repressor, thereby allowing transcription from the p_L promoter of pTrxFus.

DNA techniques. Chromosomal GAS DNA was prepared by the method of Martin et al. (26). Transformation of GAS strain CS101 by electroporation followed the protocol of Caparon and Scott (4). Preparation of plasmid DNA from *E. coli* and conventional techniques for DNA analysis and manipulations as well as PCR experiments were performed as outlined previously by Ausubel et al. (1). The generation and use of a λ library of chromosomal CS101 DNA and the isolation and sequencing of desired recombinant λ clones were done as described previously by Podbielski et al. (35).

For identification of DNase-expressing recombinant λ clones, the NZY agar was supplemented with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), calf thymus DNA, and methyl green (see above). Thus, recombinant GAS DNase genes could be expressed from the inducible P_{lac} promoter contained in the λ DNA, and resulting DNases could be detected by clear agar zones around the respective λ phages.

For DNA sequencing and sequence analysis, oligonucleotide primers were designed with the aid of the OLIGO 5.0 software (National Bioscience, Plymouth, Minn.) and were prepared as outlined previously by Podbielski et al. (34). Sequences were compiled and analyzed by utilizing the PCGENE 6.85 software (IntelliGenetics, Mountain View, Calif.). Sequences were compared with the GenBank entries by using the BLAST programs.

For cloning of the streptococcal DNase gene *sdaD* into the *Xba*I and *Pst*I sites of pTrxFus, *sdaD* was amplified by PCR by using the primers shown in Table 1. Appropriate restriction sites were added as 5′ extensions to the primers. In order to simplify the purification of the recombinant SdaD protein, the C-terminal primer contained an additional $(GTG)_6$ in-frame extension which added a poly-(His) tail to the resulting recombinant protein.

RNA techniques. RNA preparation from selected GAS strains, RNA analysis using denaturing agarose gel electrophoresis, Northern (RNA) blotting, and hybridizations with digoxigenin (Dig)-dUTP-labeled PCR products were performed as described previously by Podbielski et al. (34). The primers used for generation of the PCR products are shown in Table 1.

TABLE 1. Oligonucleotides used for cloning and generation of probes

Oligonucleotide designation[a]	Sequence (5′ to 3′)	Position no.[b]
DNA 17	TAA TAG CCG ATA GCT TAG	873–890
DNA 18	TAC CAA TAT ACA TTA GAC	1354–1337
TRX-FOR	ATC GGC CAC TAT TTC	421–435
TRX-REV	TTT TTG TAA AGA GAT ATT TTC	1309–1289
DNA-PFW1	ATC ATA ACT GTG TCT CCT GTG	2–22
DNA-PFW2	CAC TTG GCT CTG GTT TGC	710–693
DNA-PFW3	AGC AGT GGG CTG TCT AAT	1325–1342
DNA-PFW4	CTA TTG ATT TAT TGA TGT TGT TTG	2116–2096

[a] Oligonucleotides were designed with the aid of the OLIGO 5.0 software. DNA 17 and DNA 18 were used as PCR primers to generate a directly Dig-dUTP-labeled probe. TRX-FOR and TRX-REV carried an *Xba*I site and a *Pst*I site plus $(GTG)_6$ as 5′ extensions and served as PCR primers to clone the major part of *sdaD* into the pTrxFus vector. DNA-PFW1 through DNA-PFW4 carried *Bam*HI, *Xba*I, *Pst*I, and *Pin*AI sites as 5′ tags and were utilized to clone PCR-amplified fragments of the *sdaD* sequence into the pFW3 vector (37) to enable allelic replacement mutagenesis.

[b] Sequence positions numbers refer to the *sdaD* sequence presented in this study.

Protein techniques. Proteins from GAS culture supernatants were prepared by sedimenting the cultures that had been grown overnight for 10 min at 6,000 × *g* and subsequent filtration of the supernatant through 0.2-μm-pore-size membranes. The filtrate was then 30-fold concentrated by passage through Centricon 10 columns (Amicon, Witten, Germany) and stored at 4°C until further use.

Recombinant DNase was hyperexpressed from pTrxFus plasmids by 4 h of incubation of recombinant *E. coli* GI724 or GI698 at 30°C or room temperature, respectively, in 50 ml of induction medium (Invitrogen) supplemented with 100 mg of tryptophan per liter. Cells then were sedimented by centrifugation and kept frozen until further use.

Cell pellets were stirred at room temperature in 5 ml of 0.1 M sodium phosphate–0.01 M Tris buffer adjusted to pH 8.0 and containing 8 M urea–1% Triton X-100 until lysis was complete. The lysates were either directly loaded onto Ni-nitrilotriacetic acid (NTA) spin columns (Qiagen, Hilden, Germany) or dialyzed against 50 mM phosphate buffer (pH 8.0)–300 mM NaCl and then loaded onto the columns. Washing and elution of the His-tagged recombinant DNase were performed according to the manufacturer's protocol.

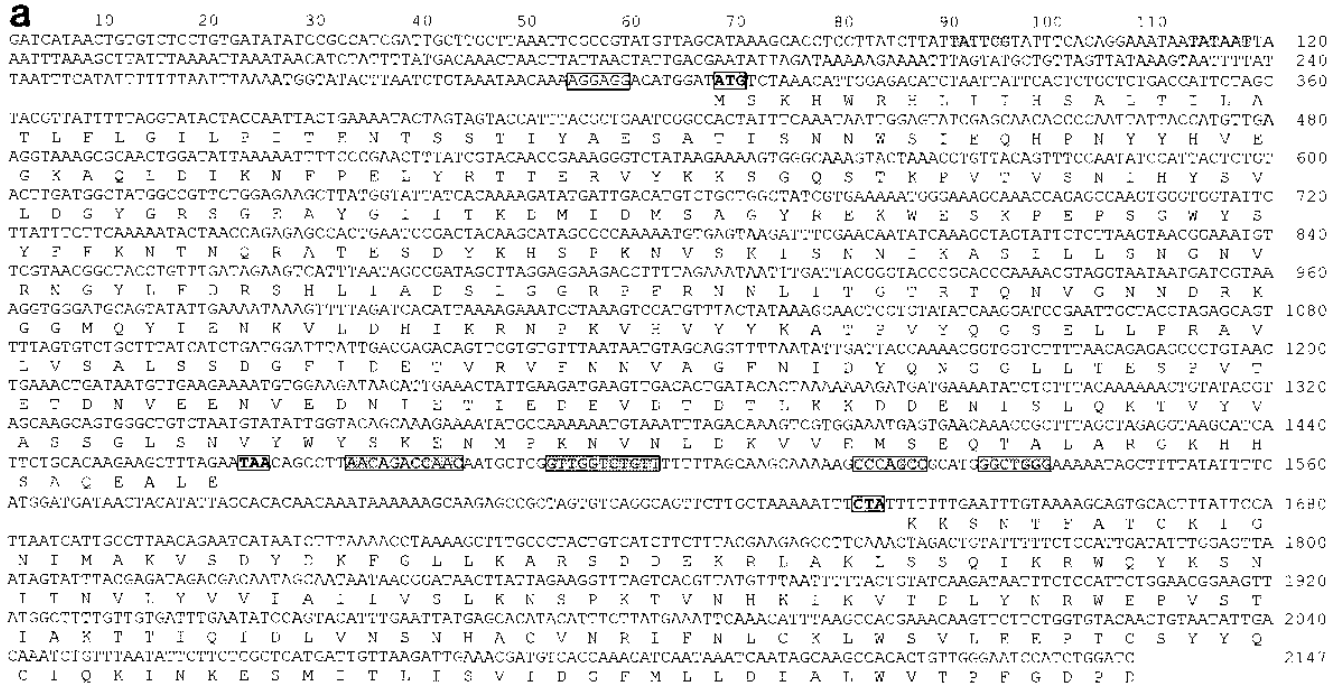
Aliquots containing 5 to 18 μg of protein from culture supernatants or preparations of the recombinant DNase were applied to 12% polyacrylamide gels. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent semidry electrotransfer onto polyvinylidene difluoride membranes were performed as described previously by Podbielski et al. (33).

Ouchterlony double diffusion assays in 1% agarose–phosphate-buffered saline (pH 7.2) utilizing aliquots containing 5 to 20 μg of protein from culture supernatants or preparations of recombinant protein and 20 μl of each DNase type-specific rabbit antiserum as well as subsequent drying and staining with Coomassie blue were performed by the protocol of Ausubel et al. (1).

Western blot (immunoblot) membranes were incubated with a Ni-NTA-alkaline phosphatase conjugate (Qiagen) directed to the His tail of recombinant DNase, with rabbit antisera specific for the four serotypes of GAS DNases, or with patient sera. Visualization was performed with chemiluminescent CSPD (Tropix-Serva, Heidelberg, Germany) and, if appropriate, a secondary antibody-alkaline phosphatase conjugate according to the instructions of the manufacturer.

The rabbit antisera were generously provided by P. Ferrieri, Minneapolis, Minn. The patient sera were obtained from the Serological Diagnostics Laboratory of the University Hospital Aachen. Anti-streptolysin O (ASL) titers and anti-DNase B (ADB) titers were determined by using latex microagglutination (Behring, Marburg, Germany) and nephelometric measurements (Nephelometer 100; Behring).

Allelic replacement mutagenesis. The strategy and techniques for inactivation of the GAS *sdaD* gene by allelic replacement with a recombinant pFW3 plasmid were described previously by Podbielski et al. (37). Briefly, two fragments comprising parts of the *sdaD* gene were amplified by PCR. The primers, as shown in Table 1, carried appropriate restriction sites as 5′ extensions. The fragments were cloned into the *Bam*HI and *Xba*I sites and *Pst*I and *Pin*AI sites of the two multiple cloning sites of pFW3. The recombinant plasmid pFW3 was electroporated into GAS strain CS101. By using spectinomycin for selective pressure, the appropriate portions of recombinant pFW3 were established in the GAS chromosomal DNA by a homologous double crossover event. A 615-bp section of the



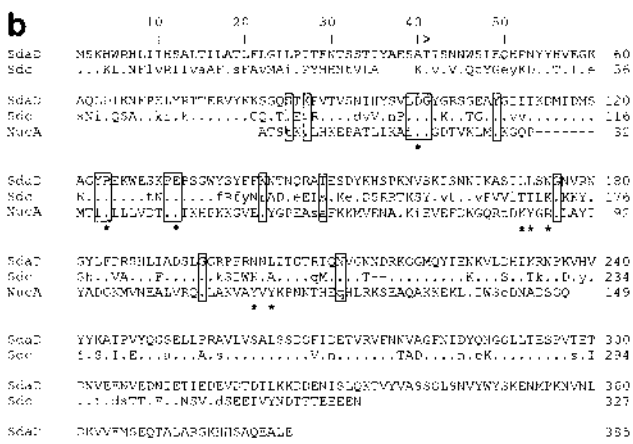


FIG. 1. (a) Nucleotide sequence and deduced amino acid sequence of the GAS DNase D. The complete nucleotide sequence of a 2,147-bp fragment of GAS genomic DNA encoding DNase D and the corresponding deduced amino acid sequence are shown. Downstream of the DNase D (*sdaD*) gene is the 3′-terminal portion of another ORF on the corresponding strand. The start and stop codons of the DNase gene and ORF are indicated by the boxes. The Shine-Dalgarno sequence upstream of the DNase D gene is indicated by the box. The potential transcription termination sites of the DNase gene and ORF are shown as stippled boxes, and the potential −35 and −10 promoter boxes and the transcription start of the DNase D gene are shown as stippled areas. (b) Alignment of the deduced amino acid sequences of the GAS DNase D (SdaD), a group C streptococcal DNase (Sdc), and *S. aureus* mature thermostable nuclease (NucA). The sequences of SdaD (this study), Sdc (50), and mature NucA (40) were aligned with the aid of the PC GENE PALIGN program. Identical residues in Sdc and NucA are indicated (.). Similar residues in Sdc and NucA are shown as lowercase letters. Gaps (–) were introduced into the Sdc and NucA sequences for maximum homology. The potential N terminus of the mature SdaD sequence is marked by an arrowhead. Residues that are similar or homologous in all three sequences are shown boxed. Residues involved in the formation of the NucA active site (7, 8, 17, 39) are marked by asterisks.

sdaD gene was thereby replaced by the *aad*9 spectinomycin resistance gene of pFW3. The specific integration of the *aad*9 gene in the chromosome was confirmed by Southern blot hybridization and PCR experiments as described previously by Podbielski et al. (37).

Assessment of DNase activity. For measurement of biochemical characteristics of SdaD, protein preparations from culture supernatants (35) were stabilized by the addition of 1 volume of glycerol or 1 volume of neopeptone broth (Difco, Detroit, Mich.) and stored at 4°C until used in amounts of 5 to 10 μg of total protein per assay. DNase activity was quantitively measured by utilizing calf thymus DNA as the substrate and methyl green indicator in liquid assays as described previously by Nelson et al. (32) and Ferreira et al. (10). The pH dependence of DNase activity was assessed by adjusting the assay buffer to pH values between 4 and 11. Inhibition by bacterial tRNA was measured upon addition to total RNA prepared from the M49 wild-type strain to a final concentration of 60 μg per liter (47).

Measurement of phagocytosis resistance. Assessment of GAS phagocytosis resistance in whole human blood by classical direct bactericidal assays and by FACScan flow cytometry followed the protocols previously outlined by Podbielski et al. (36).

Nucleotide sequence accession number. The sequence of the GAS *sdaD* gene was submitted to the EMBL data library and has been assigned the accession number X89235.

RESULTS

Sequence analysis of the *sda* gene. By using an M49 GAS-derived λ library with cloned genomic fragments which ranged in size from 2 to 9 kb, 28 of about 30,000 recombinant phages tested were found to exhibit DNase activity in the presence of IPTG inducer. Six of these phages were isolated, and the cloned inserts were partially sequenced and found to contain recombinant sequences homologous to portions of the *sdaD* sequence. By transfection, the recombinant DNA of one of these phages was established as a plasmid pBK derivative in the extracellular-DNase-negative *E. coli* XLOLR strain. This *E. coli* isolate was designated strain 1394. When cultured on DST agar supplemented with DNase activity indicator, this strain exhibited extracellular DNase activity. The 2,147-bp GAS-derived DNA from strain 1394 was completely sequenced.

Upon analysis, this sequence was found to contain one complete open reading frame (ORF) and another partial ORF composed of 1,155 and 504 bp, respectively (Fig. 1a). When comparing the entire 2,147-bp sequence to the GenBank entries, only the 1,155-bp ORF exhibited significant homology to

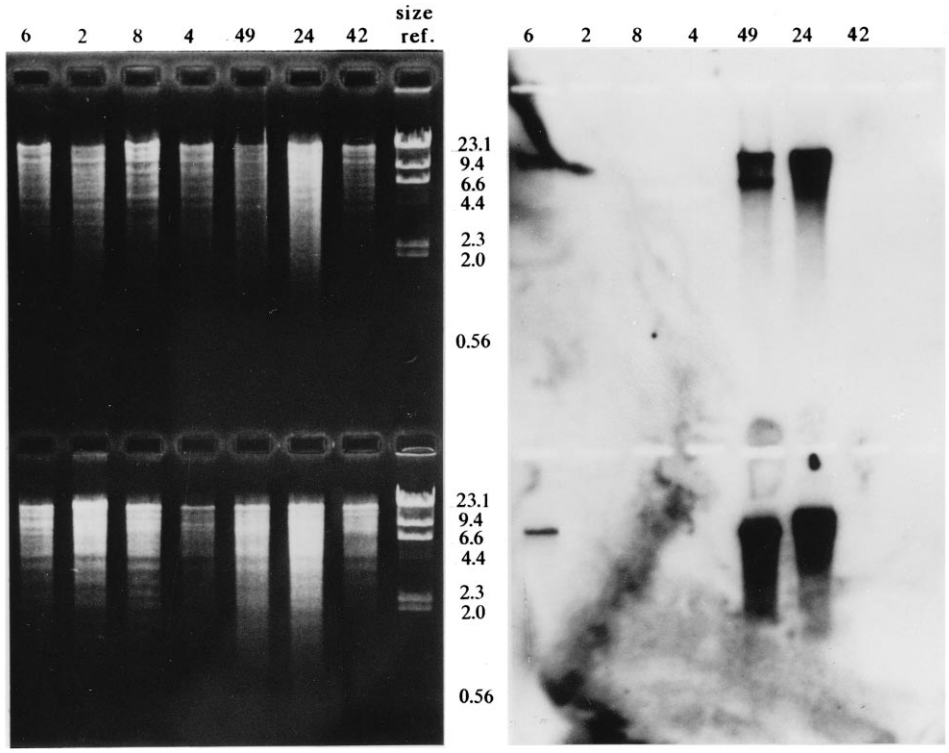


FIG. 2. Screening for the presence of the DNase D (*sdaD*) gene in GAS of selected M types. Genomic DNAs (left) from GAS of M types 2, 4, 6, 8, 24, 42, and 49 were subjected to enzymatic restriction with *Eco*RI (top) and *Xba*I (bottom). Resulting fragments were separated by agarose gel electrophoresis and stained with ethidium bromide. The size references (size ref.) (in kilobases) run in parallel are given. After Southern blotting (right), an *sdaD*-specific probe was used for hybridization. Hybridization products for M types 6, 24, and 49 were nonradioactively visualized with CSPD.

the *sdc* gene encoding the *Streptococcus equisimilis* H46A nuclease (50). Neither the 1,155-bp ORF nor the partial 504-bp ORF showed notable homology to any other GenBank entry. The comparison of the deduced amino acid sequences of the 1,155-bp ORF and the *sdc* gene exhibited 48% sequence identity and 63% sequence similarity with the GAS-derived sequence, which contained 58 more residues (385 versus 327) than the Sdc sequence (Fig. 1b). The divergent residues clustered in the potential leader peptide sequence, in a central portion of about 80 residues, and in the C-terminal parts of the sequences. On the basis of this sequence homology, the 1,155-bp GAS-derived ORF was presumed to encode a GAS nuclease and was tentatively termed *sda* (for streptococcal DNase of group A streptococci).

Further sequence analysis of the recombinant 2,147-bp fragment demonstrated the presence of a Shine-Dalgarno box as well as potential −10 and −35 boxes approximately 200 bp upstream of the *sda* start codon (Fig. 1a). Immediately downstream of *sda*, there are two inverted repeats, which could change the local entropy by −73 and −72.2 kJ/mol, respectively. The first inverted repeat is followed by a poly(T) run on the *sda*-encoding strand, while the second exhibits a poly(T) run on the corresponding strand (Fig. 1a). Thus, the inverted repeats demonstrate features of Rho-independent transcription terminators and could serve as such to end transcription of either *sda* or the partial ORF on the corresponding strand.

Utilizing oligonucleotides DNA 17 and DNA 18 (Table 1) as primers for PCR, the genomic DNAs from eight different randomly selected GAS serotype strains were screened for the presence of an internal *sda* fragment. Only in strains of serotypes M6, M24, and M49 could the 480-bp fragment be detected (Fig. 2). An identical result was obtained when the *Eco*RI- or *Xba*I-digested genomic DNA from the eight strains was Southern blotted and hybridized with a Dig-dUTP-labeled PCR product generated with primers DNA 17 and DNA 18 on serotype M49 genomic DNA template (Fig. 2).

Transcript analysis of the *sda* gene. Ten micrograms of total RNA from serotype M49 strain CS101 and twofold serial dilutions of the RNA were subjected to Northern blot hybridization, again using the DNA 17- and DNA 18-generated PCR product as a probe. Only one strong band of about 1.3 kb in size could be visualized in a minimum amount of 1.3 μg of total RNA (Fig. 3). Thus, compared with other virulence genes (34), the *sda* transcript appeared to be strongly expressed. Its size indicated a monocistronic mode of transcription and corresponded to the locations of the potential promoter and transcription terminator sites described above (Fig. 1a). A similar experiment done with total RNA from the positive regulator *mga*49 mutant (34) resulted in a single hybridization band with a size and intensity identical to those of the M49 wild type (not shown).

Characterization of an *sda* allelic replacement mutant. To enable future analysis of the biological functions of a GAS DNase, an isogenic M49 strain CS101 mutant with an inactivated *sda* gene had to be created. Since the *sda* gene was relatively small and the active sites in the encoded Sda nuclease were unknown, allelic replacement mutagenesis rather than insertional inactivation was chosen as the strategy for genetic manipulation of *sda*.

A double recombinant pFW3 plasmid, containing the *aad*9 gene flanked by an upstream and downstream portion of *sda*, was used to exchange a 615-bp portion of the *sda* gene for the

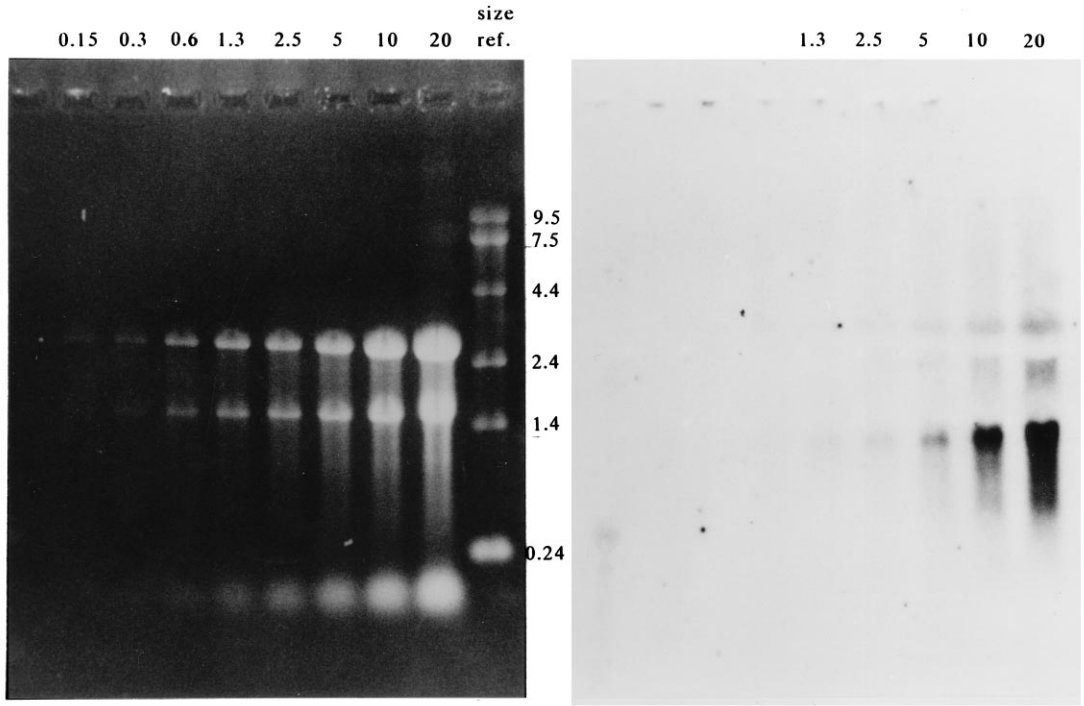


FIG. 3. Transcription analysis of the GAS DNase D (*sdaD*) gene. Serial dilutions of total RNA from M49 GAS strain CS101 were subjected to denaturing agarose gel electrophoresis (left). The amounts (in micrograms) of total RNA applied to single lanes are given over the gel. The size references (size ref.) (in kilobases) are shown. After Northern blotting (right), specific *sdaD* transcripts were detectable by hybridization if at least 1.3 μg of total RNA was used.

*aad*9 resistance gene by a double crossover recombinational event. After the presence and precise location of the mutation had been demonstrated by Southern blot hybridization and PCR (37), the *sda* mutant was subjected to several functional tests.

Using Northern blot hybridization, total RNA from the *sda* mutant was examined for the presence of an *sda* transcript. No specific *sda*-derived RNA could be visualized under conditions which allowed detection of the positive wild-type control (data not shown).

The GAS wild type and the *sda* mutant were simultaneously cultured on Todd-Hewitt agar supplemented with DNase activity indicator (for details, see Materials and Methods). Although growth of both isolates was inhibited, potentially by the methyl green supplement, only the minute colonies of the wild-type strain were surrounded by a cleared zone of DNA lysis (Fig. 4).

Protein preparations from culture supernatants of the M49 wild-type and *sda* mutant strains were subjected to Ouchterlony immunodiffusion analysis using polyclonal DNase type-specific rabbit antisera. Using culture supernatants from the M49 wild-type strain, precipitation bands were visible with the anti-type B and anti-type D antisera. By employing culture supernatants of the *sda* mutant, only the anti-type B derived band could be visualized (Fig. 5).

When Western blots of the two different culture supernatants were used for immunodetection with the four type-specific antisera, several bands ranging in size from 80 to 10 kDa were found to react with type B, C, and D antisera. This indicated that these antisera were not specific enough to be used for such a sensitive technique. Only faint bands corresponding to low-molecular-weight polypeptides were visible when anti-DNase A antiserum was used, indicating that this type of DNase was not expressed by M49 wild-type bacteria (data not shown).

Protein preparations from culture supernatants of the M49 wild-type strain and the *sda* mutant were assessed for biochemical characteristics of the Sda DNase as described in Materials and Methods. Only in protein preparations from the M49 wild-type strain could DNase activity be detected exhibiting maximum values at pH 7.0. The DNase activity was weakly inhibited upon the addition of bacterial RNA. These features were consistent with biochemical characteristics described for GAS DNase D (45, 47). Taken together, these data indicated that the investigated DNase was the type D DNase of GAS, and the encoding gene was termed *sdaD*.

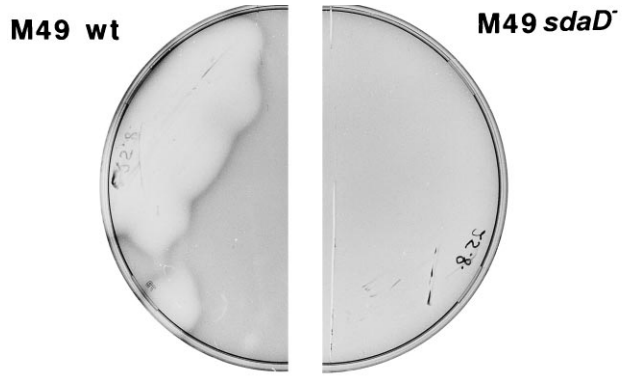


FIG. 4. DNase activity of the M49 GAS wild type (wt) and an isogenic *sdaD* mutant. Both strains were cultured on Todd-Hewitt agar supplemented with DNA and methyl green as indicators of DNase activity. Only the minute wt colonies were surrounded by a cleared zone of DNA lysis.

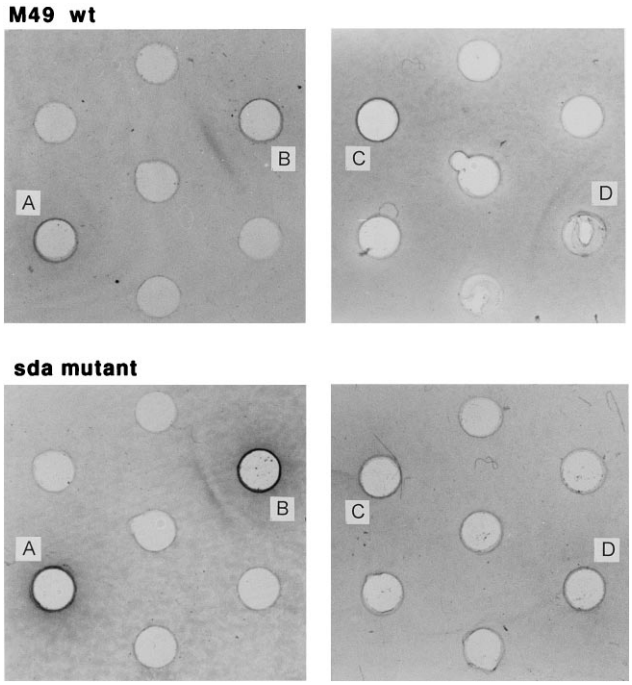


FIG. 5. GAS DNase typing by Ouchterlony immunodiffusion. Protein preparations from culture supernatants of the M49 GAS wild type (wt) and an isogenic *sdaD* mutant were subjected to Ouchterlony immunodiffusion using the four type-specific anti-GAS DNase rabbit antisera (DNase types A, B, C, and D). For improved visualization, precipitation bands were stained by Coomassie blue staining. Judging from the presence or absence of precipitation bands, the M49 wild-type strain expressed type B and D DNases while the isogenic mutant expressed only the type B DNase.

Both the wild-type and *sda* mutant bacterial strains were subjected to measurements of phagocytosis resistance. No difference could be measured between the two GAS isolates, regardless of whether activity was tested by classical direct bactericidal assays or by FACScan flow cytometry.

Preparation and characterization of the recombinant Sda protein. In order to characterize some biochemical and immunological properties of the SdaD polypeptide, the PCR-amplified major portion of the *sdaD* gene was cloned into the pTrxFus expression vector. The recombinant *sdaD* fragment was completely sequenced to confirm its integrity. The recombinant *sdaD* gene was then hyperexpressed in *E. coli*, and subsequently attempts were made to purify it by affinity chromatography of the C-terminal poly(His) tail to immobilized Ni^{2+} ions.

Upon SDS-PAGE, Western blotting, and reaction of denatured proteins from the *E. coli* host with Ni-alkaline phosphatase conjugate, three major bands with molecular masses of 48, 36, and 12 kDa could be detected. These bands corresponded to the full-size recombinant Sda protein and some C-terminal poly(His) tail breakdown products (data not shown).

Attempts to purify the recombinant Sda protein via Ni-NTA affinity columns failed. The recombinant protein did not firmly adhere to the column material and was constantly eluted with the washing buffers whether nondenatured protein preparation was in a phosphate buffer and the preparation was denatured in 6 M guanidinium HCl or 8 M urea buffer or whether denatured protein preparations were dialyzed against phosphate buffer prior to application onto the affinity column.

For confirmation of the results from immunodiffusions with culture supernatants, the protein preparation from the *E. coli* host was subjected to Ouchterlony assays as described above. Only with the anti-type D antiserum was a major precipitation band visible (data not shown).

In order to separate the recombinant protein from *E. coli* proteins and thereby reduce the background in Western immunoblots with the human sera, the denatured protein preparation was subjected to SDS-PAGE and Western blotting. The full-size recombinant SdaD protein was located on the Western blots by cutting out two lanes of each Western blot, reacting with the Ni-alkaline phosphatase conjugate, and aligning the lanes with the remainder of the Western blots. Small strips carrying the full-size recombinant Sda protein were cut out of the Western blots and were used for immunoreactions with human sera.

Presence of anti-DNase D antibodies in human sera. In order to assess the presence of anti-SdaD antibodies in human sera, strips of Western-blotted recombinant SdaD were incubated with patient sera obtained from the Serological Diagnostics Laboratory of the Aachen University Hospital.

Altogether serum samples from 40 different persons with clinical signs attributable to recent GAS infections were employed in this analysis. Each serum sample was tested for ASL and ADB titers and was assigned to one of three groups: (i) high ASL titers, (ii) high ADB titers, and (iii) low ASL and ADB titers. In a fourth group, serum samples from 10 persons with no signs of recent streptococcal infections were taken as a negative control. The patient age range, median age, and median ASL and ADB titers of each group are shown in Table 2.

Each serum sample was used in dilutions of 1:200, 1:500, and 1:1,000 for immunoreaction with the recombinant SdaD. While about half of the serum samples from patients exhibiting high ASL or ADB titers also reacted with the SdaD protein, only four serum samples from the low-titer group showed reactivity (Table 2). Four of the six anti-SdaD positive sera from groups with high ASL or ADB titers exhibited immunoreactivity to SdaD only at a 1:200 dilution, while the two other sera were also SdaD reactive at a 1:1,000 dilution.

DISCUSSION

Although thoroughly investigated on a general level, GAS DNases had not been previously characterized on a molecular basis. Therefore, the specific biological functions of these enzymes and their importance for bacterial virulence were un-

TABLE 2. Western blot immunodetection of anti-SdaD antibodies in selected serum samples from patients

Group[a]	No. of serum samples from patients	Age (yr) of patients		ASL titer[b]	ADB titer[b]	No. of serum samples with anti-SdaD titer of:	
		Range	Median			≥200	≥500
1	14	6–76	30	935	152	6	2
2	11	9–62	28	255	574	6	2
3	15	5–72	32	<93	<148	4	1
4	10	4–82	27	<105	<136	1	0

[a] Patients of groups 1, 2, and 3 exhibited clinical signs indicating a recent GAS infection (sore throat, purulent sputum, pustulae, oligoarthritis, rheumatic fever, or acute glomerulonephritis). Patients of group 1 predominantly exhibited high ASL titers, those of group 2 predominantly exhibited high ADB titers, and those of group 3 exhibited low ASL and ADB titers. Group 4 was the normal control group.

[b] ASL and ADB titers for groups 1 and 2 are given as median values, while the titers for groups 3 and 4 are given as maximum values.

known. The present study reports the first molecular characterization of a GAS DNase.

This Sda DNase was identified as a type D enzyme according to the classification of Wannamaker and Yasmineh (47). It exhibited notable homology only to the Sdc DNase of group C streptococci, reflecting the evolutionary relatedness of these bacteria. Still, the two DNases are different enough that the SdaD DNase could be actively expressed in *E. coli*, while active Sdc was not produced by this host (50). Although there was no overall homology to the well-characterized staphylococcal nucleases, alignment with the NucA sequence from *S. aureus* revealed the residues of SdaD which potentially participate in forming the enzyme's active site (Fig. 1b). Eight residues have been described to form the active site of staphylococcal nuclease A (7, 8, 17, 39). Only three of these residues appeared to be conserved in the N termini of SdaD and Sdc (Fig. 1b). This could explain functional differences between these enzymes (15).

Besides differences in their putative signal peptides and central domains, the C termini of SdaC and Sdc were found to be especially diverse in sequence and size. As predicted by computer analysis, the calculated molecular mass of mature SdaD was 39 kDa. This clearly exceeded the 25- to 30-kDa size of the DNase D protein estimated from nondenaturing electrophoresis (47). However, the binding of type-specific antibodies, the pattern of biochemical activities, and the presence of the gene in less than half of the serotypes tested identified the investigated DNase as type D DNase of GAS. Since the DNase type-specific antiserum reacted with exoproteins of different sizes from the M49 GAS wild-type strain, the discrepancy in size could also result from measurements of biochemically active breakdown products of DNase D in the former study. In support of this, Wolinowska et al. (50) have reported that Sdc breakdown products retained full activity. An alternative explanation would be that native SdaD is processed completely differently than regular secreted GAS proteins. A second potential protease signal was predicted at residue 121 (motif, . . .MSA′). This would generate a mature protein with a molecular mass of 29.9. kDa. The second cleavage site would correspond to the processing of staphylococcal nuclease, which also exhibits an unusually large leader peptide (29, 40).

DNase B has been described in several studies as being expressed by virtually all GAS strains (31, 44, 47), and on the basis of the results of the Ouchterlony assays, DNase B was also expressed by this study's GAS strain. However, in the present investigation, the only DNase identified of several λ clones recombinant with M49 GAS genomic DNA was the SdaD enzyme. This result could be a consequence of the approach chosen to identify GAS DNases as follows. (i) The DNase B gene could be part of an operon larger than the exclusion size for recombinant genomic fragments. (ii) The polypeptide could need processing by GAS-specific proteins not encoded in proximity to the DNase B gene. In fact, potential glycosylation or dimerization of DNase B has been observed in former studies (15, 44). (iii) The expression of DNase B by the M49 *sdaD* mutant and the simultaneous complete lack of exo-DNase activity could indicate that the method used for screening the DNase activity did not detect a DNase B-related nuclease activity, although this method has been successfully used for DNase B detection (10, 44). (iv) Finally, these results could also indicate that in the investigated strain CS101, type D enzyme was the only active DNase produced while DNase B remained inactive under the chosen culture and screening conditions. This aspect would contribute to another observation that production of different types of DNases, in particular DNase B, could be serotype or even strain specific in GAS (31). Reevaluation of the former data did not yield clear results, since the epidemiology of the four types of DNases in relation to the M types of the investigated strains had not been fully recorded.

Although SdaD was thoroughly characterized on the molecular level, its contribution to the strain's virulence remained unclear. In vitro mRNA analysis demonstrated that *sdaD* was transcribed at comparatively high levels. Its transcription was clearly not influenced by the Mga regulator, which has been shown to control several virulence factors in strain CS101 (36). RofA, the only other regulatory protein described so far for GAS (12), could not be tested for its influence on SdaD expression, since it was not encoded in this M49 isolate. Also, specific stimuli for regulation remain to be determined. The presence of substrate in culture medium did not stimulate DNase production (45). The presence of SdaD-specific antibodies in human sera confirmed the immunogenicity of SdaD. The fact that about 50% of patients with elevated ASL and/or ADB titers also exhibited anti-SdaD antibodies indicated that such antibodies were regularly induced by GAS infections. The percentage of SdaD antibody-positive patients correlates with the observations that less than 50% of the GAS strains investigated did produce DNase D (44) and less than half of the GAS strains tested in this study carried the *sdaD* gene. Anti-SdaD antibodies could serve as an additional marker for GAS infections, since these antibodies were also detectable in ASL- and ADB-negative GAS-infected patients. As judged by experiments with human granulocytes, SdaD did not contribute to the bacteria's resistance to phagocytosis. Because of its mode of activity, SdaD should primarily contribute to GAS invasiveness into host tissue. This virulence feature of SdaD could be hard to demonstrate in an in vitro assay with tissue cultures. From the fact that staphylococcal nuclease activity in a mouse infection model became detectable mainly at the end of the infection (6), it can be inferred that expression of DNase is regulated by complex interactions between bacteria and host not encountered in tissue culture models. Now, the molecular characterization of SdaD in conjunction with the availability of novel GAS vectors with green fluorescent protein reporters (37) should easily allow for in vivo measurements (41) to finally elucidate the importance of GAS DNase in bacterial virulence.

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