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Effects on Virulence of Mutations in a Locus Essential for Hyaluronic Acid Capsule Expression in Group A Streptococci

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Mucoid or highly encapsulated strains of group A streptococci have been associated both with unusually severe infections and with acute rheumatic fever. Previously, we described an acapsular mutant, TX4, derived from a mucoid M-type 18 strain of a group A streptococcus by transposon mutagenesis (M. R. Wessels, A. E. Moses, J. B. Goldberg, and T. J. DiCesare, Proc. Natl. Acad. Sci. USA 88:8317–8321, 1991). We now report studies further characterizing strain TX4 as well as an additional acapsular mutant, TX72. Strain TX4 was found to contain a 9.5-kb deletion of chromosomal DNA adjacent to the site of transposon Tn*916* insertion. Cloned chromosomal DNA from TX4 flanking the transposon insertion site was used as a probe to demonstrate the presence of homologous regions in 11 of 11 wild-type group A streptococcal strains of various M protein types. A second acapsular mutant, TX72, had a single transposon insertion and had no apparent deletion of chromosomal DNA. The Tn*916* insertion in TX72 was mapped to the *hasA* locus (encoding hyaluronate synthase), which lies within the chromosomal region deleted in TX4. Strain TX72 was avirulent in mice and sensitive to phagocytic killing in vitro. Transduction of either the insertion-deletion mutation from TX4 or the simple insertion mutation from TX72 to a type 24 group A streptococcus strain also resulted in loss of capsule expression, demonstrating that a homologous region of the chromosome controls capsule expression in another serotype of group A streptococci. We conclude that the hyaluronic acid capsule plays an important role in virulence and that a region of the chromosome essential for capsular polysaccharide expression is conserved among diverse group A streptococcal strains.

Group A streptococci (GAS) are the etiologic agents of streptococcal pharyngitis and impetigo, two of the commonest bacterial infections in children, as well as a variety of less common but potentially life-threatening infections, including soft tissue infections, bacteremia, and pneumonia. In addition, GAS are uniquely associated with the postinfectious autoimmune syndromes of acute rheumatic fever and poststreptococcal glomerulonephritis. Several recent reports suggest that the incidence both of serious infections due to GAS and of acute rheumatic fever has increased during the past decade, focusing renewed interest on defining the attributes of the organism that may play a role in the pathogenesis of these diseases (6, 26, 29, 31, 33).

GAS produce several surface components and extracellular products that may be important in virulence. The major surface protein, M protein, has been studied in the most detail and has been shown convincingly to play a role in both virulence and immunity. Isolates rich in M protein are able to grow in human blood, a property thought to reflect the capacity of M protein to interfere with phagocytosis, and these isolates tend to be virulent in experimental animals (20, 27).

Clinical observations have suggested that the hyaluronic acid capsule also may be important in virulence: mucoid, or highly encapsulated, strains are uncommon among clinical isolates of GAS in general, but appear to be proportionally more frequent among isolates associated with invasive infections or acute rheumatic fever. In a survey of more than 1,100 GAS isolates collected in the United States between 1988 and 1990, Johnson et al. (13) found that only 3% of pharyngitis isolates were mucoid. In contrast, 21% of the strains associated with invasive infections were mucoid, and 42% of the isolates associated with acute rheumatic fever were mucoid (13). Although some ascertainment bias in the collection of these strains cannot be excluded, the strikingly increased prevalence of mucoidy among invasive and rheumatic fever-associated isolates suggests an association between capsule expression and virulence. Until recently, experimental evidence supporting the role of the hyaluronic acid capsule of GAS in virulence has been less well defined. Studies in the 1940s by Kass and Seastone (14) showed that the virulence of encapsulated streptococci could be attenuated by treatment with hyaluronidase, but other investigators, using different methods, failed to demonstrate a virulence role for the capsule (12).

To better define the role of the GAS capsule in pathogenesis and to understand the mechanisms through which the capsular polysaccharide may serve a virulence function, we and others have begun to characterize a region of the GAS chromosome essential for capsule expression and to analyze the effects of mutation within this region on virulence. Our previous studies of TX4, an acapsular mutant derived from a mucoid (i.e., highly encapsulated) strain of GAS by transposon mutagenesis, demonstrated that, in contrast to the wild-type strain, the acapsular mutant was unable to grow in human blood, was sensitive to phagocytic killing in 10% human serum, and was relatively avirulent in mice (32).

Dougherty and van de Rijn subsequently reported that TX4, the acapsular mutant used in our earlier studies, and a similar mutant derived in their laboratory harbored a deletion of chromosomal DNA of undetermined size adjacent to the site

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid (source of clinical isolate)	Relevant features[a]	Source or reference
Escherichia coli JM107	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*ΔM15/*endA1 gyrA96* (Nal^r) *thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA*	35
Enterococcus faecalis CG110	Tn*916* donor strain	11
Streptococcus pyogenes		
87-282 (Ohio)	M type 18, mucoid (Cap$^+$), clinical isolate (rheumatic fever)	E. Kaplan (32)
282S1	M type 18, mucoid (Cap$^+$), Sm^r derivative of 87-282	32
TX4	M type 18, nonmucoid (Cap$^-$), Tc^r, contains Tn*916* and 9.5-kb chromosomal deletion, derived from 87-282	32
TD27	M type 18, nonmucoid (Cap$^-$), Tc^r, contains two Tn*916* insertions, derived from 282S1	This report
TX72	M type 18, nonmucoid (Cap$^-$), Tc^r, derived from 87-282 by transduction of single Tn*916* mutation from TD27	This report
SS90	M type 3	R. Facklam
SS799	M type 3	R. Facklam
87-136 (Ohio)	M type 3, nonmucoid	E. Kaplan
86-764 (Utah)	M type 3, mucoid	E. Kaplan
86-346 (Utah)	M type 18, nonmucoid	E. Kaplan
9001 (Massachusetts)	Clinical isolate (pharyngitis)	This laboratory
9002 (Tennessee)	Mucoid, clinical isolate (rheumatic fever)	K. Edwards
22967 (Massachusetts)	Clinical isolate (necrotizing fasciitis)	J. Parsonnet
Vaughn	M type 24, mucoid (Cap$^+$)	M. Bronze
24-4	M type 24, nonmucoid (Cap$^-$), Tc^r, contains Tn*916* and 9.5-kb chromosomal deletion, derived from Vaughn	This report
24-72	M type 24, nonmucoid (Cap$^-$), Tc^r, contains Tn*916*, derived from Vaughn	This report
CS24	M type 12	P. Cleary
Plasmids		
pAM120	21.2 kb, contains Tn*916*, Ap^r Tc^r	11
pUC18	2.7 kb, Ap^r	35
pMW6T	pUC18 with 20.7-kb *Eco*RI-*Bam*HI::Tn*916* insert, Ap^r Tc^r	This report
pMW6	pUC18 with 1.8-kb *Eco*RI-*Bam*HI insert, Ap^r (derived from pMW6T by spontaneous excision of Tn*916*)	This report

[a] Abbreviations: Ap, ampicillin; Cap, capsular polysaccharide phenotype; Nal, nalidixic acid; Sm, streptomycin; Tc, tetracycline.

of Tn*916* insertion (9). This finding raised the possibility that the avirulence of strain TX4, previously attributed to the absence of capsule expression, might actually be due to the loss of another virulence factor encoded within the deleted segment of the GAS chromosome. Subsequent investigations revealed that the segment deleted in TX4 included *hasA*, the gene encoding hyaluronate synthase, and *hasB*, encoding UDP-glucose dehydrogenase (8–10); both enzymes are involved in hyaluronic acid biosynthesis.

We now report studies that define the extent of the chromosomal deletion in strain TX4, demonstrate conservation of the capsule gene region in multiple strains of GAS, and confirm the role of the capsule in pathogenesis through virulence studies of an acapsular mutant harboring a single transposon insertion in *hasA* with no associated chromosomal deletion.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. GAS strains were grown in Todd-Hewitt broth or on Todd-Hewitt agar containing 5% defibrinated sheep blood. *Escherichia coli* was grown in Luria-Bertani broth or on Luria-Bertani agar (25). Antibiotics were added to culture media for some experiments as follows: ampicillin, 50 μg/ml; tetracycline, 5 μg/ml; and streptomycin, 500 μg/ml. For cloning experiments, *E. coli* JM107 was grown on agar supplemented with isopropyl-β-D-thiogalactopyranoside (40 μg/ml) and 5-bromo-4-chloro-3-indolyl-D-galactoside (40 μg/ml) (Sigma Chemical Company, St. Louis, Mo.) to permit identification of white colonies resulting from insertional inactivation of the β-galactosidase gene on the cloning vector, pUC18.

Antisera. M protein type 18 antiserum was prepared in rabbits by intravenous injection of Formalin-fixed organisms of GAS strain 87-282. The specificity of this serum for type 18 M protein has been demonstrated previously (32). Type 24 antiserum was a gift of Michael Bronze, University of Tennessee, Memphis.

Isolation of mutants. Transposon Tn*916* was transferred from *Enterococcus faecalis* donor strain CG110 by filter mating, and nonmucoid transconjugants were isolated as described previously (32). Briefly, equal numbers of cells of donor and recipient strains were combined and then deposited on membrane filters. The filters were incubated overnight, and the bacteria were removed and plated on medium containing tetracycline and streptomycin. Culture plates were screened by visual inspection for nonmucoid colonies.

Bacteriophage transduction. Bacteriophage transduction was performed by the method of Caparon and Scott (5). Bacteriophage A25 (provided by June Scott, Emory University, Atlanta, Ga.) was used to infect a broth culture of the nonmucoid transconjugant TD27. A lysate of this culture was filtered free of bacterial cells and then transferred to a broth culture of wild-type strain 87-282. Transductants that had incorporated DNA from TD27 containing a Tn*916* insertion were selected on tetracycline agar. Tetracycline-resistant transductants were screened by inspection for the nonmucoid colony phenotype.

Transduction of the transposon mutation from TX4 or TX72 to wild-type strain Vaughn was accomplished by infecting a broth culture of either mutant strain with bacteriophage A25, transferring a lysate to a culture of strain Vaughn, and selecting tetracycline-resistant transductants as described above.

DNA manipulations. Genomic DNA was isolated from GAS strains as described by O'Connor and Cleary (21). Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (2), as described elsewhere (25). Larger amounts of plasmid pMW6 were purified with the pZ523 Spin Column kit according to the manufacturer's instructions (5 Prime-3 Prime, Inc., Boulder, Colo.). DNA fragments prepared by restriction enzyme digestion were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide (25). When required, individual fragments were excised from the gel and purified by using a commercially available kit (GeneClean [Bio 101, La Jolla, Calif.] or Sephaglas BandPrep Kit [Pharmacia]).

DNA-DNA hybridizations. After electrophoresis, DNA fragments were transferred by capillary blotting from the agarose gel to a nylon membrane (Hybond N+ [Amersham, Arlington Heights, Ill.] or Duralon [Stratagene, La Jolla, Calif.]). Southern hybridization analysis was performed by two methods: as described previously (32) with DNA probes labeled with [α-^{32}P]dCTP by the random-primed hexamer extension method according to the manufacturer's instructions (Random Primed DNA Labeling Kit; Boehringer Mannheim Biochemicals, Inc., Indianapolis, Ind.) and with the enhanced chemiluminescence system (ECL; Amersham), according to the manufacturer's instructions. The first method was used for the experiment shown in Fig. 5; the second method was used for those shown for Fig. 2, 3, and 4. For some experiments (Fig. 5), the entire pMW6 plasmid was used as a probe. In other experiments (Fig. 2 and 4), the probe was prepared by digesting pMW6 with *Eco*RI and *Bam*HI and then purifying and labeling the 1.8-kb fragment as described above. The entire pAM120 plasmid (containing Tn*916*) was used as a probe to identify Tn*916* insertions in GAS mutants (Fig. 3).

Cloning. TX4 genomic DNA was digested to completion with the restriction enzymes *Eco*RI and *Bam*HI, which do not cut within the Tn*916* sequence. The digested TX4 DNA was ligated to *Eco*RI- and *Bam*HI-digested pUC18 and transformed into *E. coli* JM107 as described before (25). Colonies that grew on tetracycline-ampicillin agar were subcultured in liquid medium containing ampicillin without tetracycline. Subsequent subcultures of the organisms grown in the absence of tetracycline confirmed loss of tetracycline resistance, indicating excision and loss of the Tn*916* sequence from the recombinant plasmid. An *Eco*RI-*Bam*HI digest of this plasmid, designated pMW6, yielded fragments of 2.7 kb, corresponding to the pUC18 vector, and 1.8 kb, representing the cloned TX4 DNA.

DNA sequencing. DNA sequencing of the termini of the pMW6 insert was performed by the dideoxy chain termination method with the Sequenase version 2.0 kit, M13 (−40) sequencing primer 5′-GTTTTCCCAGTCACGAC-3′, and M13 reverse primer 5′-AACAGCTATGACCATG-3′ (United States Biochemical Corporation, Cleveland, Ohio). Plasmid pMW6 was denatured in 0.2 M NaOH for 10 min at room temperature for use as the template in sequencing reactions. After denaturation, plasmid DNA was recovered by precipitation with 1/10 volume of 3 M sodium acetate, pH 5.3, plus 2.5 volumes of ethanol at −20°C overnight. DNA was pelleted at 13,000 × *g* for 10 min, dried briefly under vacuum, and then dissolved in water for immediate use in sequencing reactions. Sequencing reactions, polyacrylamide gel electrophoresis, and autoradiography were performed according to the manufacturer's recommendations.

PCR. Primers were synthesized complementary to sequences near the right end (TNR0; 5′-TGAGTGGTTTT GACC-3′) and left end (TNL0; 5′-GTGAAGTATCTTCT TAC-3′) of Tn*916*, each reading outward from the transposon, as described by Clewell et al. (7), and complementary to sequences near either end of the 1.8-kb insert cloned in pMW6, MW6U1 (5′-ATTGGAGTAGTTCATCC-3′), and MW6R1 (5′-GTGACTGCTATTTACG-3′), each reading inward from the ends of the insert. PCR was carried out in a total volume of 50 μl, containing 500 ng of chromosomal DNA as the template, 25 pmol of each oligonucleotide primer, 200 μM each dATP, dCTP, dGTP, and dTTP (Pharmacia), 10 mM Tris-HCl (pH 8.3), 5 mM KCl, 1.5 mM $MgCl_2$, and 2 U of *Taq* polymerase (Cetus Corporation, Norwalk, Conn.). Samples were denatured at 94°C for 5 min, and then 35 cycles of amplification were carried out with a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, Conn.). Cycling parameters were denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min. After cycling was completed, samples were held at 72°C for 5 min to complete extension of the PCR products.

Phenotypic characterization of GAS strains. Cell-associated hyaluronic acid was measured by the carbazole method (15) after the capsule was released from the bacteria with chloroform, as described previously (32). M protein extracts were prepared by the method of Lancefield (19). Immunodiffusion in agarose was performed by the method of Ouchterlony (22).

Virulence studies of GAS strains. Whole-blood phagocytic assays (direct bactericidal test of Lancefield) were performed essentially as described before (18). Opsonophagocytic assays in 10% human serum were performed by a modification of the method of Baltimore et al. (1). Bacterial cells from a logarithmic-phase broth culture were mixed with leukocytes (6×10^6) purified from freshly drawn human blood in 0.5 ml of minimal essential medium containing 10% normal human serum as a complement source. Aliquots (25 μl) were withdrawn for quantitative culture immediately and after 1 h of end-over-end rotation at 37°C. Mouse lethality studies were performed as described previously (32) with female CD1 mice, each weighing 20 to 30 g. Briefly, 10-fold dilutions of logarithmic-phase organisms were administered by intraperitoneal injection to groups of six mice. Mortality was determined 3 days after challenge. The 50% lethal dose (LD_{50}) was calculated for each strain from results of two independent experiments including a total of 60 animals for each strain (24).

RESULTS

Cloning of DNA flanking the Tn*916* insertion in acapsular mutant strain TX4. Southern hybridization analysis of genomic DNA from acapsular mutant strain TX4 indicated the presence of a single copy of transposon Tn*916* in the TX4 chromosome (32). In order to clone DNA that flanked the transposon insertion from TX4 (for use as a probe in subsequent experiments), we used the strategy developed by Gawron-Burke and Clewell for cloning genes interrupted by Tn*916* mutagenesis (11): a fragment of chromosomal DNA containing the Tn*916* insertion was cloned by using tetracycline resistance as a selectable marker. Because Tn*916* is unstable in *E. coli*, culture of the *E. coli* strain carrying the recombinant plasmid in the absence of tetracycline resulted in excision of Tn*916* from the recombinant plasmid, leaving the vector and the DNA that flanked Tn*916* in the mutant chromosome. To use this approach, we cloned a 20.7-kb *Eco*RI-*Bam*HI frag-

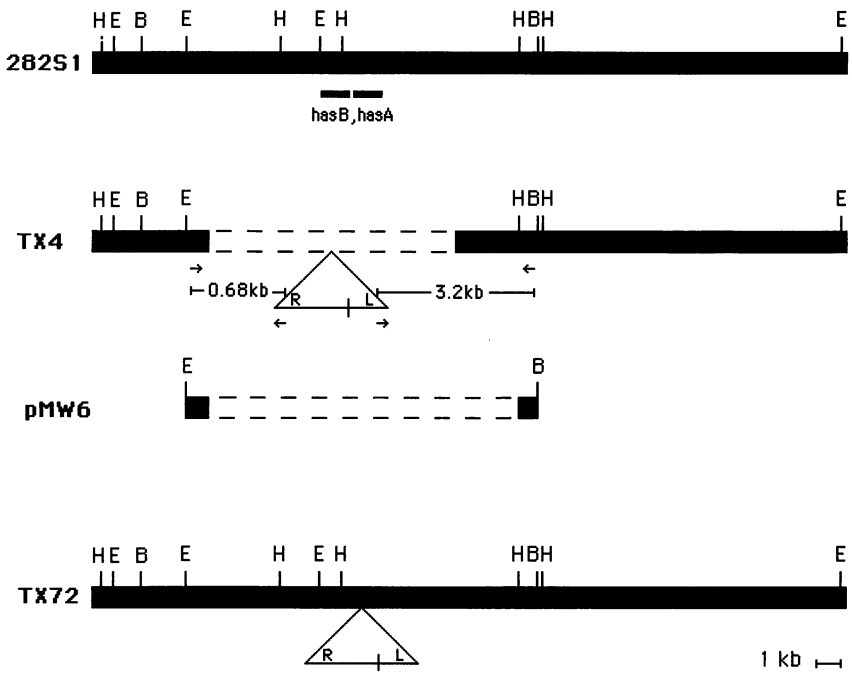

FIG. 1. Partial chromosomal restriction maps of GAS strain 282S1 and of acapsular mutants TX4 and TX72. The locations of the capsule synthesis genes *hasA* and *hasB* are indicated (8–10). The sites of Tn*916* insertion in strains TX4 and TX72 are indicated by triangles, with the position of the internal *Hin*dIII site within the transposon sequence designated by a vertical line. Dashed lines represent the 9.5-kb chromosomal deletion adjacent to the site of Tn*916* insertion in TX4. Plasmid pMW6T was generated by ligation of *Eco*RI- and *Bam*HI-digested pUC18 to the *Eco*RI-*Bam*HI fragment of TX4 chromosomal DNA that included Tn*916*; passage of transformants harboring the recombinant plasmid in the absence of tetracycline yielded plasmid pMW6. Intervening DNA separating the two parts of the pMW6 insert in the 282S1 chromosome is indicated by dashed lines. All known *Bam*HI restriction sites are indicated by B; all known *Eco*RI sites are indicated by E. All *Hin*dIII sites discussed in the text are indicated by H, although additional sites may also be present but not shown. Sequences complementary to PCR primers used to map the deletion in strain TX4 are indicated by arrows, and the size of the PCR product amplified by each pair of primers is shown. From left to right, primers were MW6R1 (left end of pMW6, reading inward), TNR0 (right end of Tn*916*, reading outward), TNL0 (left end of Tn*916*, reading outward), and MW6U1 (right end of pMW6, reading inward). The designations of the left and right ends of Tn*916*, indicated in the figure by L and R, respectively, refer to published sequence assignments (7) and are opposite to the orientation of Tn*916* in this figure.

ment of TX4 DNA that included the entire Tn*916* transposon. Analysis of *Eco*RI-*Bam*HI restriction digests of plasmid DNA from cells harboring the resultant plasmid, pMW6T, showed a 2.7-kb fragment, corresponding to the pUC18 vector, and a 20.7-kb fragment, corresponding to the expected Tn*916*-containing insert, but also a 1.8-kb digestion fragment. We speculated that this unanticipated 1.8-kb fragment arose from spontaneous loss of DNA, including the Tn*916* sequence, from a portion of the recombinant plasmids, even in the presence of tetracycline. When cells harboring pMW6T were subcultured in liquid medium containing ampicillin but not tetracycline, the tetracycline resistance was lost. Plasmid preparations of cells that had lost tetracycline resistance contained only a 4.5-kb plasmid, designated pMW6, consisting of a 2.7-kb *Eco*RI-*Bam*HI fragment corresponding to the pUC18 vector and the 1.8-kb insert (Fig. 1). These results are consistent with the expected excision and loss of the Tn*916* sequence from the recombinant plasmid in the absence of tetracycline selective pressure (11). Excision of Tn*916* may not be completely precise but generally restores the flanking sequences to within a few bases (4, 7). Therefore, excision of the 16.4-kb Tn*916* sequence from pMW6T should have left approximately 4.3 kb of flanking insert DNA in pMW6. That the size of the residual flanking insert in pMW6 was only 1.8 kb suggested that Tn*916* excision from the recombinant plasmid was accompanied by a concomitant deletion of approximately 2.5 kb of the flanking insert DNA present in pMW6T.

Mapping the region of the Tn*916* insertion in TX4. Genomic DNA from wild-type strain 282S1 or from acapsular mutant strain TX4 was digested with several restriction enzymes, singly or in combination, and then examined by Southern blot hybridization with pMW6 as a probe. The pMW6 probe hybridized with a single *Bam*HI fragment of approximately 16 kb in 282S1 and with a fragment larger than 23 kb in TX4 (Fig. 2), indicating that Tn*916* had inserted within the 16-kb *Bam*HI fragment. Plasmid pMW6 hybridized to two *Eco*RI fragments in 282S1 (5 and 22 kb) but to a single high-molecular-weight fragment in TX4. The presence of an internal *Eco*RI site in the chromosomal DNA of 282S1 hybridizing to pMW6 was not expected, since pMW6 was cloned from a complete *Eco*RI-*Bam*HI digest of TX4 DNA. The probe hybridized to two *Hin*dIII fragments in 282S1 and in TX4, a common 1-kb fragment and a second fragment of 7 kb in 282S1 and 16 kb in TX4. The anticipated increase in size of the 7-kb *Hin*dIII fragment after Tn*916* insertion would be 5.4 or 10.9 kb, the sizes of the two *Hin*dIII fragments of Tn*916*; however, the observed increase in size was 9 kb. This discrepancy in anticipated versus observed size of the fragments hybridizing to the probe was also observed in double digests of TX4 with *Hin*dIII and either *Eco*RI or *Bam*HI (Fig. 2). These experiments suggested that the two flanks of Tn*916* represented by the 1.8-kb fragment cloned in pMW6 were actually separated by several kilobases in the 282S1 chromosome and that much of the intervening DNA was deleted in TX4, including an

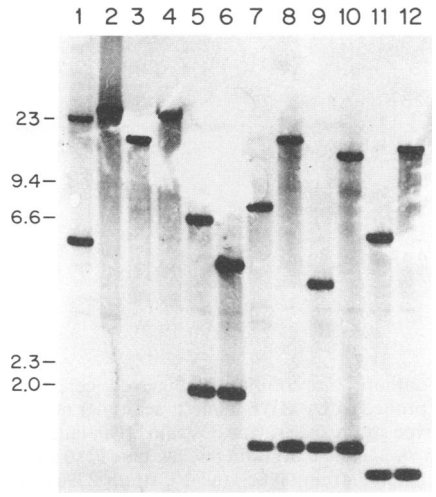

FIG. 2. Southern blot of restriction digests of genomic DNA from wild-type strain 282S1 (odd-numbered lanes) and acapsular mutant strain TX4 (even-numbered lanes) probed with the 1.8-kb insert of GAS DNA from plasmid pMW6. Restriction enzymes used were *Eco*RI (lanes 1 and 2), *Bam*HI (lanes 3 and 4), *Hin*cII (lanes 5 and 6), *Hin*dIII (lanes 7 and 8), *Eco*RI plus *Hin*dIII (lanes 9 and 10), and *Bam*HI plus *Hin*dIII (lanes 11 and 12). The migration of molecular size standards (lambda *Hin*dIII fragments) is indicated (in kilobases) on the left.

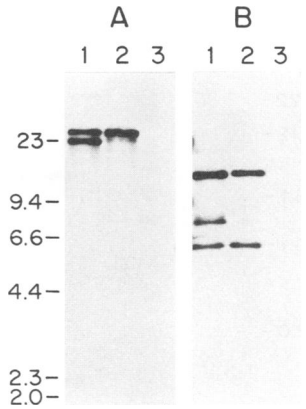

FIG. 3. Southern blot of *Eco*RI (A) and *Hin*dIII (B) digests of genomic DNA from acapsular transconjugant TD27 (lanes 1), acapsular transductant TX72, derived from TD27 (lanes 2), and wild-type strain 282S1 (lanes 3), probed with the Tn*916*-containing plasmid pAM120. The Tn*916* sequence contains no *Eco*RI sites and a single *Hin*dIII site, so each transposon insertion results in a single *Eco*RI fragment and two *Hin*dIII fragments hybridizing to the probe. Although only three bands are clearly distinguishable in the TD27 lanes, one band presumably represents two fragments of similar molecular size. This occult doublet is probably represented by the highest-molecular-weight band in TD27, as this band is broader and more intense than the other fragments in TD27 but not in TX72, in which only one of the comigrating fragments has been transduced. The migration of molecular size standards (lambda *Hin*dIII fragments) is indicated (in kilobases) on the left.

*Eco*RI site and a *Hin*dIII site. Figure 1 shows a partial restriction map of this region of the 282S1 and TX4 chromosomes, including the site of transposon insertion and the associated chromosomal deletion in TX4.

The presence and extent of the deletion in TX4 were confirmed by PCR. Primers complementary to sequences in the right and left ends of Tn*916* and to sequences near the right and left ends of the 1.8-kb insert in pMW6 were synthesized. PCR with primers that annealed 60 bp from the right end of Tn*916* and 340 bp from the left end of the 1.8-kb pMW6 insert amplified a 0.68-kb fragment from TX4 chromosomal DNA, indicating that the right end of Tn*916* was approximately 0.96 kb from the *Eco*RI site corresponding to the left end of the pMW6 insert (Fig. 1). A 3.2-kb fragment was amplified by using primers 90 bp from the left end of Tn*916* and 240 bp from the right end of the 1.8-kb fragment, indicating that the left end of Tn*916* was approximately 3.3 kb from the *Bam*HI site corresponding to the right end of the pMW6 insert. Together with the Southern hybridization experiments, these results indicated that, during transposon mutagenesis, TX4 had lost approximately 9.5 kb of chromosomal DNA within the region bounded by the two parts of the 1.8-kb fragment (Fig. 1). In addition, assuming that the left side of the pMW6 insert contained the entire 0.96-kb fragment to the left of the transposon insertion in TX4, the right side of the pMW6 insert contained only 0.8 kb of the 3.3 kb of flanking DNA to the right of Tn*916* in the TX4 chromosome. Therefore, approximately 2.5 kb of TX4 chromosomal DNA on the right flank of the Tn*916* insertion must have been lost during cloning of pMW6.

Derivation of another acapsular transposon mutant, strain TX72. The observation that strain TX4 had lost 9.5 kb of chromosomal DNA adjacent to the site of Tn*916* insertion suggested that the loss of capsule expression might be due not to the interruption of gene expression by the transposon sequence, but rather to deletion of a capsule gene(s) contained within the missing segment. Since *hasA* and *hasB*, two genes known to function in capsule expression (8–10), were contained within the deleted region, other transposon insertions resulting in the acapsular phenotype might be expected to map to this region as well. To test this hypothesis, we examined another acapsular mutant derived from strain 282S1 by Tn*916* mutagenesis. This mutant, TD27, had two Tn*916* insertions. The insertion responsible for the absence of capsule expression was identified by transducing the mutation into wild-type strain 87-282 to create a single-insertion acapsular mutant, TX72. Southern blots of *Hin*dIII-digested DNA from TX72 showed hybridization of the Tn*916* probe to two junction fragments of 14.5 and 6.3 kb. These fragments in TX72 corresponded to two of the four *Hin*dIII fragments that hybridized to Tn*916* in TD27, indicating incorporation of the mutation in TX72 by homologous recombination, without transposition of Tn*916* (Fig. 3).

Mapping the site of Tn*916* insertion in TX72. To determine whether the transposon insertion in TX72 was within the region of the chromosome that was deleted in TX4, Southern blots of TX72 *Bam*HI or *Eco*RI digests were probed with pMW6. The probe hybridized to a single high-molecular-weight *Bam*HI fragment, indicating the transposon insertion was within the 16-kb fragment bounded by *Bam*HI sites in the 282S1 chromosome (Fig. 4). The *Eco*RI digest showed hybridization of the probe to the same 5-kb fragment as in the wild-type 282S1 and to a fragment larger than 22 kb, indicating insertion of the transposon within the 22-kb *Eco*RI fragment of 282S1. The pattern of hybridization to a *Hin*dIII digest of TX72 was identical to that for 282S1, indicating that the Tn*916* insertion in TX72 lay between the internal *Hin*dIII sites of 282S1. These results localized the transposon insertion in TX72 to the 8-kb region in 282S1 bounded by the *Eco*RI site contained within the TX4 deletion on the left side and by the right internal *Hin*dIII site on the right side (see Fig. 1). The

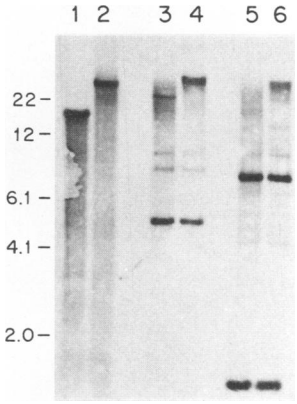

FIG. 4. Southern blot of restriction digests of genomic DNA from wild-type strain 282S1 (odd-numbered lanes) and acapsular mutant strain TX72 (even-numbered lanes) probed with the 1.8-kb insert of GAS DNA from plasmid pMW6. DNA was digested with *Bam*HI (lanes 1 and 2), *Eco*RI (lanes 3 and 4), or *Hin*dIII (lanes 5 and 6). In lanes 3 to 6, in addition to the two distinct bands in each lane, additional faint bands representing partial digestion products are visible. The migration of molecular size standards (22-kb lambda *Bgl*II fragment and 1-kb DNA ladder; BRL) is indicated (in kilobases) on the left.

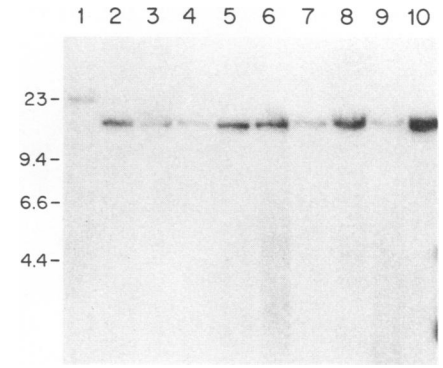

FIG. 5. Southern blot of *Bam*HI-digested genomic DNA from GAS strains probed with pMW6. Lane 1, acapsular mutant strain TX4; lane 2, wild-type strain 282S1; lane 3, strain SS90; lane 4, strain 87-136; lane 5, strain 86-346; lane 6, strain 86-764; lane 7, strain SS799; lane 8, strain 9001; lane 9, strain 9002; lane 10, strain 22967. Similar results were obtained with two additional wild-type strains, Vaughn and CS24 (not shown). The migration of molecular size standards (lambda *Hin*dIII fragments) is indicated (in kilobases) on the left.

sizes of the restriction fragments hybridizing both with the Tn*916* probe and with the pMW6 probe were those anticipated for the corresponding 282S1 fragments, indicating that Tn*916* insertion in TX72 was not associated with any deletion of chromosomal DNA detectable by these methods.

To localize more precisely the site of Tn*916* insertion in TX72, we used PCR to amplify the segment of chromosomal DNA extending from the right end of the transposon to the chromosomal *Eco*RI site to the left of the transposon insertion site (Fig. 1). To amplify this segment, TX72 chromosomal DNA was digested with *Eco*RI, ligated to *Eco*RI- and *Hin*dIII-digested pUC18, and used as a template for PCR with the M13/pUC18 reverse primer and oligonucleotide TNR0 as the second primer (annealing to the right end of Tn*916*, reading outward from the transposon). A 1.8-kb fragment was amplified (not shown), indicating that the transposon insertion site lay approximately 1.7 kb to the right of the chromosomal *Eco*RI site (Fig. 1). This result localizes the Tn*916* insertion site in TX72 within the *hasA* gene, based on the published restriction maps of the *hasA* locus (8, 10). As further confirmation, *Hin*dIII digestion cleaved the PCR product into two fragments, each approximately 0.85 kb in size (not shown), as predicted by the same restriction maps.

Hybridization of pMW6 to genomic DNA from other GAS strains. To investigate whether the chromosomal region involved in capsule expression in the mucoid (highly encapsulated) strain 282S1 was present in other GAS strains, we probed Southern blots of genomic DNA from 11 different strains of GAS representing both mucoid and nonmucoid isolates, four different M protein types, and strains isolated from patients with asymptomatic pharyngeal colonization, uncomplicated pharyngitis, acute rheumatic fever, and invasive infection. In all strains, pMW6 hybridized to a single 16-kb *Bam*HI fragment (Fig. 5). Similarly, the probe hybridized to the same two *Eco*RI fragments as in 282S1 (data not shown), except in a single strain in which the internal *Eco*RI site appeared not to be present, resulting in hybridization of the probe to a single high-molecular-weight *Eco*RI fragment. These results suggest that this region of the chromosome is relatively conserved among diverse GAS strains despite differences in capsule phenotype, M protein expression, and association with various disease states.

Transduction of capsule mutations to an M protein type 24 strain. The pMW6 probe hybridized to a similar restriction fragment in several isolates of GAS, suggesting that the chromosomal region required for capsule expression in strain 282S1 was conserved among GAS strains. To confirm that this locus was required for capsule expression for other strains as well, we transduced the transposon mutation from either strain TX4 or TX72 to the M protein type 24 strain Vaughn to produce transductants 24-4 and 24-72, respectively. Southern blots of restriction digests of both transductants demonstrated insertion of the transposon at the same site as in the type 18 mutants (not shown). Both 24-4 and 24-72 grew as nonmucoid colonies, and neither produced detectable amounts of cell-associated hyaluronic acid (<5 mg/g of cell protein, compared with 66 mg of cell-associated hyaluronic acid per g of cell protein for wild-type strain Vaughn). Ouchterlony immunodiffusion gels with type-specific antisera showed that extracts of the type 24 transductants (and the wild type) expressed type 24 and not type 18 M protein. These results indicate that a gene(s) in this chromosomal region is also essential for capsule expression in another serotype of GAS.

Phenotypic characterization of mutant strain TX72. The absence of capsule expression in strain TX72 was confirmed by measuring the amount of uronic acid released from the surface of the organisms after treatment with chloroform. Wild-type strain 282S1 yielded 73 mg of cell-associated hyaluronic acid per g of cell protein, while none (<5 mg) was detected from strain TX72. To assess M protein expression by TX72, M protein extracts were prepared as described by Lancefield (19). The extract from TX72 reacted with M protein type 18 antiserum to form a line of identity with an extract from wild-type strain 282S1 in Ouchterlony immunodiffusion gels (data not shown). The quantity of M protein produced by the acapsular mutant strain TX72 was not reduced but actually appeared to be slightly greater than that produced by the wild-type strain: a precipitin line was visible at a 1:2 dilution and faintly visible at 1:4 for the extract from TX72 but was only visible at a 1:2 dilution for strain 282S1. The rate of growth, expression of group A carbohydrate, and hemolysis on sheep blood agar were similar for TX72 and 282S1 (data not shown).

TABLE 2. Growth of GAS strains 282S1 and TX72 in blood

Strain and expt no.	CFU[a]		Increase	
	Initial	After 3 h	Fold	Log_{10} CFU
282S1				
1	1.3×10^3	1.8×10^4	14	1.1
2	4.5×10^3	2.9×10^4	6.4	0.81
3	1.8×10^4	3.2×10^5	18	1.2
Mean ± SD			12.8 ± 5.8	1.0 ± 0.20
TX72				
1	3.5×10^3	1.3×10^3	−0.37	−0.43
2	5.2×10^3	9.6×10^3	1.8	0.27
3	1.4×10^4	4.8×10^4	3.4	0.54
Mean ± SD			1.6 ± 1.9	0.13 ± 0.50

[a] Values represent means for quantitative cultures of duplicate samples.

Virulence testing of acapsular mutant strain TX72. We showed previously that acapsular mutant strain TX4 was sensitive to phagocytic killing, as reflected by failure to grow in human blood and by sensitivity to killing in an in vitro assay of opsonophagocytosis by human leukocytes in the presence of 10% nonimmune serum (32). Testing strain TX72 yielded results similar to those with TX4 in both assays. Like strain TX4, TX72 grew little or not at all in human blood, while the wild-type strain 282S1 increased approximately 10-fold during a 3-h incubation (Table 2). In the presence of human leukocytes and 10% serum, the number of cells of TX72 was reduced more than 100-fold, compared with negligible reduction in wild-type strain 282S1 (Table 3). Very little reduction in viable counts of TX72 was observed when the serum was heat inactivated before incubation with bacteria and leukocytes, indicating that active serum complement was required for efficient opsonophagocytic killing.

Virulence testing in mice was performed to confirm that the sensitivity of strain TX72 to phagocytosis in vitro correlated with loss of virulence in an animal model of infection. Groups of mice were challenged with a single intraperitoneal injection of graded doses of bacteria, and survival was assessed after 3 days. As outbred mice were used for these studies, some variation in the susceptibility of different lots of animals is expected. Thus, the LD_{50} values in these experiments both for wild-type strain 282S1 and for acapsular mutant strain TX4 were higher than those reported previously (32). In the current studies, the LD_{50} for 282S1 was 1.0×10^7 CFU, compared with 1.6×10^9 CFU for TX4 and 4.2×10^9 CFU for acapsular mutant TX72. The magnitude of the difference in LD_{50} for the acapsular mutants relative to the wild-type strain in these experiments was very similar to the 150-fold difference observed previously between acapsular mutant strain TX4 and 282S1 (32). These results demonstrated that insertion of Tn*916* in the *hasA* locus resulted not only in loss of capsule expression but also in a loss of virulence equivalent to that observed with the transposon insertion-chromosomal deletion event in TX4.

TABLE 3. Opsonophagocytic killing of GAS strains in 10% human serum

Strain	Assay components[a] and expt no.	CFU[b]		Killing	
		Initial	After 1 h	%	Log_{10} CFU
282S1	WBC + C′				
	1	2.6×10^7	2.0×10^7	17	0.11
	2	9.0×10^6	7.8×10^6	13	0.062
TX72	WBC + C′				
	1	3.4×10^7	7.2×10^4	99.8	2.7
	2	6.4×10^6	4.6×10^4	99.3	2.1
	WBC + ΔC				
	1	2.9×10^7	7.6×10^6	74	0.58
	2	7.8×10^6	5.8×10^6	26	0.12
	C′ (no WBC)				
	2	6.2×10^6	1.3×10^7	0	−0.32

[a] Assay components included bacteria, human peripheral blood leukocytes (WBC) or an equal volume of medium, and 10% human serum (C′) or 10% human serum heated to 56°C for 30 min to inactivate complement (ΔC).

[b] Values represent means for quantitative cultures of duplicate samples.

DISCUSSION

We showed previously that insertion of a single copy of transposon Tn*916* within the chromosome of the highly encapsulated GAS strain 282S1 was associated with a complete absence of expression of capsular polysaccharide (32). In the current studies, we cloned DNA sequences that flanked the transposon insertion in the GAS acapsular mutant strain TX4 and used the cloned fragment to map this region of the mutant and wild-type chromosomes. The results indicate that the Tn*916* insertion in TX4 was associated with a deletion of approximately 9.5 kb of contiguous chromosomal DNA. The presence of the deletion in TX4 suggested that the acapsular phenotype of this strain might reflect the deletion of a gene(s) required for capsule expression rather than simply interruption of a required gene by the transposon. Because the segment deleted in TX4 includes *hasA*, the gene encoding hyaluronate synthase, we anticipated that other transposon mutants with defects in capsule expression might have insertions within this region. This prediction was confirmed by Southern hybridization analysis of acapsular mutant strain TX72, another Tn*916* mutant derived from the same wild-type strain as TX4. The single transposon insertion in TX72 was mapped to the segment of the GAS chromosome deleted in TX4 and further localized to the *hasA* locus. The transposon insertion in TX72, however, was not associated with any detectable chromosomal deletion.

Dougherty and van de Rijn found that a capsule-deficient GAS mutant strain (WF61) with an insertion-deletion similar or identical to that in TX4, had a transposon insertion associated with a deletion of undefined size in an adjacent segment of chromosomal DNA (9). DeAngelis et al. also found a chromosomal deletion of at least 4 kb in the same region associated with Tn*916* insertion in a capsule-deficient GAS mutant derived from another wild-type strain (8). Our current studies have defined the size of the chromosomal deletion in TX4, and presumably in WF61, since Dougherty and van de Rijn reported that the insertion-deletion in WF61 was identical to that in TX4 (9). The extent of the deletion in the mutant described by DeAngelis et al. was not defined; however, these authors did report an oligonucleotide sequence (GGGCTGA CATATTGTGCCA) flanking the transposon insertion-deletion, obtained by direct sequencing of a chromosomal restriction fragment containing Tn*916* by using a Tn*916*-specific primer (8). This oligonucleotide sequence is identical to nucleotides 874 to 892 (reading from the *Eco*RI site) in the portion of the pMW6 sequence representing chromosomal DNA from the left flank of the insertion-deletion in TX4 (unpublished results). Thus, it appears that acapsular mutants derived independently by Tn*916* mutagenesis from three different GAS strains all harbor similar or identical deletions adjacent to the transposon insertion site. The deletion of a specific segment of chromosomal DNA in association with Tn*916* insertion in three independently derived acapsular mutants suggests a

unique interaction between the transposon and this region of the GAS chromosome. The structural basis for this deletion event is not yet known.

Like the genes controlling capsule biosynthesis in group B streptococci and in gram-negative bacteria, the capsule genes of GAS appear to be clustered on the chromosome (3, 16, 17, 30). To date, the capsule gene region of the GAS chromosome has been shown to include two genes encoding enzymes involved in hyaluronic acid synthesis (8, 10). Another GAS acapsular mutant, WF62, with a Tn*916* insertion in *hasA* was found not to express the *hasB* gene product (10), suggesting that these two genes may be part of a single transcriptional unit. The observation that transposon insertion in TX4 was accompanied by a large chromosomal deletion including both *hasA* and *hasB* implies that, although a gene(s) within the deletion may be required for capsule expression, it is not essential for cell viability. Since deletion of nearly 9.5 kb of the region was compatible with apparently normal cell viability and growth, it should be possible to define more precisely the function of additional genes within this region by analyzing the effects of mutations at multiple sites within the deleted region.

GAS strains vary widely in colonial morphology from compact, nonmucoid, or "glossy" colony type to wet, spreading, mucoid, or matt colonies. This spectrum of colony appearance is due primarily to differences in the amount of capsular hyaluronic acid (34). Our results indicate that at least one region of the GAS chromosome containing genes required for capsule expression is conserved among nonmucoid as well as mucoid GAS strains. This conclusion was supported both by the results of Southern hybridization studies of multiple GAS strains and by duplication of mutations involving this region in another serotype of GAS. A possible interpretation of these findings is that the genes required for capsule synthesis are present in all GAS strains and that differences in degree of encapsulation among wild-type strains reflect regulatory differences in the expression of these or related gene products. Strains of GAS that grow as mucoid colonies when first isolated from ill patients may have diminished production of capsular polysaccharide after laboratory passage as well as diminished expression of M protein (28). In some strains, both M protein expression and the mucoid phenotype may be restored by passage in animals or by repeated culture of the organisms in human blood. These observations suggest that capsule expression may be regulated by environmental factors, although neither the environmental signals nor the genetic elements involved in the regulation have been identified. The regulatory locus located upstream from the M protein gene, *mry* or *virR*, appears not to regulate capsule expression (23).

The spontaneous diminution in M protein and capsule expression sometimes observed with laboratory passage of clinical isolates has been correlated with loss of resistance to phagocytosis and reduced virulence in animals. Insertional mutagenesis of a gene(s) required for capsule expression has made it possible to distinguish the effects of the capsule from those of M protein in these measures of virulence. Localization of the TX72 mutation to the gene encoding hyaluronate synthase strongly suggests that it is the loss of encapsulation that accounts for the avirulence of strain TX72, rather than a more global effect on one or more occult virulence factors in addition to the abrogation of capsule expression. This view is supported by the fact that we detected no other phenotypic changes (apart from capsule expression) in acapsular mutants TX4 and TX72, including expression of type 18 M protein. However, definitive proof that the avirulence of TX72 is due to the absence of capsule expression will require the development of genetic constructs to test the effects on both capsule expression and virulence of complementing the defect in TX72 with the cloned *hasA* gene product in *trans*.

Both acapsular mutants appeared to produce slightly larger amounts of M protein than the wild-type strain 282S1. It is possible that M protein extraction is more efficient in the absence of capsule, although we have shown previously that decapsulation of mucoid organisms by hyaluronidase treatment did not significantly alter the yield of M protein extracted from the bacterial cells (32). Since M protein and the capsular polysaccharide are major surface molecules of the organism, it may be that loss of capsule expression resulted in an increase in metabolic energy devoted to M protein production. Despite normal or increased levels of M protein, loss of capsular polysaccharide expression was associated with loss of resistance to phagocytosis both in whole blood and in 10% human serum. Reduced virulence in mouse lethality studies paralleled the sensitivity of the acapsular mutants to phagocytosis in vitro, providing further evidence that the hyaluronic acid capsule plays a critical role in the pathogenesis of GAS infection in vivo.

The identification of a defined region of the GAS chromosome as one required for capsular polysaccharide production will facilitate future studies to elucidate the molecular mechanisms controlling capsule expression. Definition of the molecular basis for capsule expression ultimately may lead to a better understanding of the relationship between the mucoid phenotype and streptococcal diseases.

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