Molecular Characterization of a Locus Required for Hyaluronic Acid Capsule Production in Group A Streptococci

By Brian A. Dougherty and I. van de Rijn

From Wake Forest University Medical Center, Winston-Salem, North Carolina 27157

Summary

To characterize the production of hyaluronate capsule by the membrane-associated enzyme hyaluronate synthase (HAS), group A streptococci from a recent outbreak of acute rheumatic fever were mutagenized via Tn*916* insertion. Acapsular transconjugants harboring multiple, nontandem copies of the transposon were identified and found to lack HAS activity (<1% of wild-type levels). Generalized transduction was then performed to determine which Tn*916* insertion was responsible for the HAS^- phenotype. These marker exchange experiments resulted in the isolation of two distinct classes of acapsular transductants, designated WF61 and WF62. Both transductants also lacked significant HAS activity, and excision of the transposon from WF62 restored capsular hyaluronate production. Southern analysis of WF61 DNA demonstrated a large deletion of genomic DNA adjacent to the Tn*916* insertion. This deletion event is presumably responsible for the observed stability of the acapsular phenotype of WF61. Further analyses of transductant whole-cell DNA indicated that the transposon insertions of WF61 and WF62 were separated by 2.5 kb. These studies define a locus required for hyaluronate capsule production in group A streptococci. Further genetic analysis of this locus has identified a gene required for HAS activity which was inactivated by Tn*916* in WF62 and deleted in WF61.

Lancefield group A streptococci (*Streptococcus pyogenes*) are human pathogens capable of causing a variety of infections, as well as nonsuppurative sequelae such as acute rheumatic fever and poststreptococcal glomerulonephritis. Many strains of both groups A and C streptococci are able to surround themselves with a capsule of hyaluronate (hyaluronic acid), with the former accounting for the majority of serious human infections. Group C streptococci are primarily animal pathogens, however, and are not known to initiate postinfectious sequelae. In addition to the major virulence factor M protein, which protrudes from the surface of group A streptococci (1), the hyaluronate capsule has also been implicated as a virulence factor (2, 3).

During the last decade, a resurgence of acute rheumatic fever has been reported by several investigators (4). An enhancement of some virulence factor(s) of the streptococci could explain the severe suppurative and nonsuppurative sequelae associated with these outbreaks. One notable characteristic of these group A streptococcal isolates was their highly mucoid nature. Capsular hyaluronate is thought to endow group A streptococci with the ability to resist phagocytosis in mice (3, 5, 6). In contrast, in vitro studies demonstrated the presence of a heat-labile component present in human blood (but absent from the blood of mice and rabbits) that neutralized the antiphagocytic effect of the hyaluronate capsule (7, 8). Stollerman et al. (9) showed, however, that the sera from a small number of rabbits and humans were exceptions to this observation. More recently, Wessels et al. (2) constructed an acapsular mutant to further investigate the role of the hyaluronate capsule in human infections. These workers demonstrated, in contrast to the above studies, that the ability to survive in fresh human blood and the antiphagocytic nature of the streptococci appeared to correlate with the presence of a hyaluronate capsule. Therefore, the role of hyaluronate as a major virulence factor in humans is open to further experimentation.

Structural identity has been demonstrated between some bacterial capsules and components of mammalian tissue (10). Hyaluronate produced by groups A and C streptococci is chemically indistinguishable from that found in the connective tissue and biological fluids of mammals. This may account for the poor immunogenicity of hyaluronate (11). Antibodies that react with hyaluronate are present in low titer in both the sera of several animal species (12) and that from patients with systemic lupus erythematosus and poststreptococcal glomerulonephritis (13, 14). Furthermore, using encapsulated streptococcal or liposome-bound hyaluronate, Fillit et al. (15, 16) were able to induce a humoral immune response to hyaluronate in rabbits and mice, respectively.

Hyaluronate is a linear glycosaminoglycan composed of

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β 1–4-linked repeating disaccharide subunits of glucuronic acid β 1–3 linked to *N*-acetylglucosamine. The hyaluronate synthase (HAS)[1] enzyme system, associated with the streptococcal membrane, produces the heteropolysaccharide from the precursors uridine diphosphate glucuronic acid (UDP-GlcUA) and UDP-*N*-acetylglucosamine (UDP-GlcNAc) (17). Although cell-free synthesis was demonstrated over 35 years ago (18), an understanding of the mechanism of hyaluronate biosynthesis remains incomplete. A step towards characterizing this mechanism was achieved in the work of Triscott and van de Rijn (19) with the digitonin-solubilization of HAS activity from streptococcal membranes, and optimization of enzyme activity with the phospholipid cardiolipin. One reported purification of a group C HAS protein (20) failed to demonstrate a single purified protein with biosynthetic activity. Attempts by our laboratory to purify HAS to homogeneity have resulted in a concomitant loss of HAS activity. Therefore, final purification of an enzymatically active HAS remains elusive.

In the present study, an alternative strategy for characterization of the streptococcal HAS was devised that involves the identification and expression of the genes required for hyaluronate synthesis. Analysis of the HAS genes and construction of isogenic mutants would greatly aid in elucidating the mechanism of hyaluronate biosynthesis, and in further characterizing a streptococcal virulence factor. As a step towards these goals, mutants lacking HAS activity were isolated and characterized. These experiments define a genetic locus required for hyaluronate synthetic activity.

Materials and Methods

Bacterial Strains, Media, and Antibiotics. The bacterial strains used in these studies are listed in Table 1. Streptococci were confirmed to be of the Lancefield group A using specific antisera by precipitin assay (21). Unless otherwise indicated, streptococci were grown at 37°C in chemically defined media (CDM [22]) while *Escherichia coli* were grown by shaking at 37° in Luria Bertani (LB) broth (23). For growth of streptococci on solid media, Todd Hewitt broth (Difco Laboratories, Detroit, MI) was supplemented with 1.5% agar. Growth of bacteria was measured by OD using a Spectronic 20 (Bausch & Lomb, Inc., Rochester, NY) at a wavelength of 650 nm. All strains were maintained frozen at −80°C in media containing 50% glycerol.

When appropriate, antibiotics were used at the following concentrations: streptococci, tetracycline (5 μg/ml), streptomycin (1,000 μg/ml); *E. coli*, tetracycline (10 μg/ml), ampicillin (50 μg/ml), and chloramphenicol (25 μg/ml).

Spontaneous streptomycin-resistant group A streptococci were isolated by growing 5-ml cultures of bacteria, and plating onto media containing 100 μg/ml streptomycin. Colonies resistant to 100 μg/ml streptomycin were similary grown and plated on media supplemented with 1,000 μg/ml streptomycin. Two variants (WF14 and WF13) resistant to 1,000 μg/ml streptomycin were derived in this fashion from WF50 and WF51, respectively.

Plasmids and Purification of DNA. Plasmids used in this study include pACYC184 ([24], kindly provided by F. Macrina) Virginia Commonwealth University, Richmond, VA, pUC18 (Bethesda Research Laboratories, Gaithersburg, MD), and pBluescript II SK$^-$ (Stratagene Inc., La Jolla, CA). The chimeric plasmid pAM120 consists of Tn*916* cloned into the plasmid pGL101 (25).

Plasmids were introduced into *E. coli* by the one step transformation method (23). *E. coli* XL1-Blue (Stratagene, Inc.) transformed with recombinant pBluescript plasmids were screened after plating on LB supplemented with 50 μm isopropyl-B-D-thiogalactopyranoside (5 Prime-3 Prime, Inc., West Chester, PA), and 0.02% 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (5 Prime-3 Prime, Inc.) to detect insertional activation of the *lacZ*α gene. Plasmid DNA was purified by the alkaline lysis method (23), and DNA concentrations were determined by measuring the absorbance at 260 nm.

Whole-cell DNA was isolated from mutanolysin-treated group A streptococci. Briefly, CDM (20 ml) supplemented with 20 mM glycine was inoculated with the appropriate culture and incubated overnight. The culture was pelleted and resuspended to 500 μl total vol in TE-sucrose (10 mM tris [pH=7.2], 1 mM EDTA, and 25% sucrose). Mutanolysin (2 U/ml) and lysozyme (3.1×10^5 U/ml) were added, and the mixture was incubated at 37°C for 1 h. The cells were then lysed with *n*-lauroyl sarcosine (1% final concentration), and incubated with RNase A (20 U/ml) at 65°C for 30 min. Next, the DNA mixture was incubated with pronase (100 U/ml) for 1 h at 37°C followed by extraction with phenol and chloroform. After ethanol precipitation, the DNA was washed with 70% ethanol and resuspended in distilled water (200 μl).

Filter Matings. Tn*916* was transferred to group A streptococci as described by Franke and Clewell (26) with modifications. Briefly, cultures of the recipient group A streptococci (grown in CDM containing hyaluronidase [70 U/ml]) and the CG110 donor were mixed at a ratio of 40:1, respectively, and deposited onto a filter membrane. The filters were incubated overnight and bacteria were then plated on solid media supplemented with tetracycline and streptomycin to select for transconjugants.

Transductions. Generalized transductions were performed using the streptococcal bacteriophage A25 as described by Caparon and Scott (27). M17 broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) was substituted for the various liquid and solid media called for in the procedure. Dextrose (5% final concentration) was added to agar plates when differentiation between mucoid and nonmucoid colonies was required.

Hyaluronate Production. Qualitative production of hyaluronate capsule was monitored by India ink preparations (28). For confirmation of hyaluronate synthase activity, membranes were prepared by phage lysin treatment of group A streptococci, according to the procedure of van de Rijn (29). Enzyme solubilization and HAS activity were accomplished, as follows, by modifications of the protocols described by Triscott and van de Rijn (19).

Membranes (5 mg/ml) isolated from streptococci grown to the exponential phase were solubilized using 0.5% dodecyl-β-D-maltoside in buffer A (0.05 M $NaHPO_4/KH_2PO_4$, pH 6.9, 0.15 M NaCl, 10 mM $MgCl_2$, 5 mM dithioerythritol, 10% glycerol) at 4°C for 60 min with stirring. Insoluble membrane components were removed by sedimentation at 196,000 *g* for 1 h at 4°C. The supernatant that contained the HAS activity was then stored at −70°C until use.

Analysis of the transfer of UDP-[U-^{14}C]glucuronic acid to hyaluronic acid was monitored as follows. Membrane extracts (50 μl) were combined with reaction mixture (50 μl, containing 0.182 μmol dithioerythritol, 0.455 μmol $MgCl_2$, 0.6 μmol of UDP-GlcNAc, and 0.148 nmol of UDP-GlcUA [8.6×10^4 cpm] in 47 mM $NaHPO_4/KH_2PO_4$, pH 6.9 buffer). All reactions were performed

[1] *Abbreviations used in this paper:* CDM, chemically defined media; GlcUA, glucuronic acid; HAS, hyaluronate synthase; LB, Luria Bertani broth; ORF, open reading frame; SSP-PCR, single-specific-primer polymerase chain reaction; UDP, uridine diphosphate.

at 37°C and were initiated by the addition of enzyme protein and terminated by the addition of 10% SDS (10 μl), followed by heating at 100°C for 2 min. Reaction rates were taken from the initial linear portion of the product versus time curves and were run in duplicate.

For determination of radioactivity incorporated into the high molecular weight fraction, the reaction mixtures were loaded onto spin columns prepared from Sephadex G-50 in 1-ml plastic syringes (23). The eluted high molecular weight material was then mixed with Ecolume (ICN Biomedicals, Inc., Costa Mesa, CA), and incorporated radioactivity was quantitated using a scintillation counter. Zero time controls were run to correct for residual radioactivity.

Protein Determination. Protein quantitation was accomplished using the bicinchoninic acid assay (BCA; Pierce Chemical Co., Rockford, IL). Since buffer A contains a number of components that interfere with the BCA protein assay, all samples were treated as follows before analysis. Extracts (75 μl) were mixed with acetone (300 μl) and incubated at 4°C for 5 min. The samples were then sedimented at 13,000 *g* for 5 min and the supernatant was discarded. The samples were next treated with absolute ethanol (100 μl) at 4°C for 3 min followed by resedimentation of the precipitate and removal of the supernatant. Finally, the samples were resuspended in 0.1 N NaOH (100 μl) and analyzed for protein content. All samples were evaluated in duplicate using BSA as standard.

DNA Manipulations. DNA restriction and modification enzymes were obtained from Promega Corp. (Madison, WI) and used in accordance with the manufacturer's suggestions. DNA fragments were routinely separated by horizontal agarose gel electrophoresis and visualized by ethidium bromide staining (23). Restriction or modification of isolated DNA fragments were performed in GTG Seaplaque agarose (FMC Bioproducts, Rockland, ME) according to the manufacturer's recommendations. When required, DNA was further purified using the GeneClean kit (Bio 101, La Jolla, CA).

DNA/DNA hybridizations were performed after transferring DNA from agarose gels to Nytran membranes (Schleicher & Schuell, Keene, NH) by capillary blot (23). DNA probes were labeled with α-[^{32}P]dCTP (NEN Research Products, Boston, MA) using a random primed DNA labeling kit (Boehringer Mannheim Biochemicals, Inc., Indianapolis, IN). Hybridizations were performed using standard high stringency conditions (23). Bands were visualized by exposure of filters to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) at −80°C.

PCR Amplification of DNA Flanking Tn916. *S. pyogenes* DNA flanking Tn*916* was amplified by the single-specific-primer polymerase chain reaction (SSP-PCR) method of Shyamala and Ames (30). PCR reactions were performed in a final volume of 100 μl containing 100 ng DNA template (40 ng restriction-digested transductant whole-cell DNA ligated to 60-ng compatibly digested pUC18 DNA), 1.0 μM oligomers, 1.0 μM concentration of each deoxynucleoside triphosphates, and 10 μl *Taq* DNA polymerase 10× buffer (Promega Corp.). 2 U of *Taq* DNA polymerase (Promega Corp.) was added to the reaction mixture upon reaching the denaturing temperature of 93°C, and the amplification was carried out for 30 cycles using a DNA Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CT). The parameters for PCR consisted of denaturation (93°C) for 1 min, annealing (55°C) for 3 min, and extension (70°C) for 1 min. The plasmid-specific primer used was the M13 (−47) primer (New England Biolabs, Inc., Beverly, MA). Transposon-specific primers TNL1 (5′-TATAAAGCTTGAGAAGCAACAGGAGCGTCTTGTTGC-3′) and TNR1 (5′-TATTAAGCTTGACACCTCATTTTTTGAAGTGTCTAC-3′) were synthesized based on the sequence of the Tn*916* termini published by Clewell (31), and contained tailored HindIII restriction sites to facilitate cloning of PCR products.

DNA Library Construction and Screening. A genomic library of *S. pyogenes* DNA was prepared using the Lambda Fix II/XhoI Partial Fill-in Vector Kit (Stratagene Inc.) according to the manufacturer's protocol. Briefly, WF14 whole-cell DNA was partially digested with Sau3AI to yield restriction fragments in the range of 12–24 kb, filled in with dGTP and dATP, and ligated to the λ replacement vector arms. Recombinant phage were selected using *E. coli* P2392 (Stratagene Inc.) and recombinant clones were screened by plaque lift hybridizations (23). After two rounds of plaque purification, the phage DNA was purified using the Lambdasorb phage absorbent protocol (Promega Corp.).

Reversion to Capsule Production. Because of the reported instability of Tn*916* in the absence of selective pressure (25, 32), attempts were made to select for excision of the transposon from the locus required for capsule production. Acapsular transductants were passaged by inoculation of a loopful of glycerol stock into 5-ml liquid media lacking tetracycline. The overnight culture was then diluted and plated onto medium lacking antibiotics. After overnight incubation at 37°C, colonies were scored for capsule production. As a control the glycerol stock was diluted, plated directly onto medium lacking antibiotics, and similarly incubated and scored.

Results

Tn916 Mutagenesis of Group A Streptococci. To study capsule synthesis in these pathogenic streptococci, encapsulated *S. pyogenes* strains were mutagenized via transposon insertion. The conjugative transposon Tn*916* was transferred from the group D streptococcal strain CG110 into the streptomycin-resistant group A strains WF13 and WF14. The resultant transconjugants were selected on medium containing both tetracycline (to select for Tn*916*) and streptomycin (to select against CG110).

Transconjugants were screened for nonmucoid colonies which were then tested for capsule production by India ink staining. Four unencapsulated transconjugants (WF15, WF20, WF22, and WF35) were further characterized for this study. All acapsular mutants were confirmed to be of the Lancefield group A by precipitin reaction.

Hyaluronate Synthase Assay of Transconjugants Lacking Capsule. To ensure that loss of capsule correlated with loss of hyaluronate synthase activity, membranes from encapsulated strains and acapsular mutants were isolated, solubilized, and assayed for production of radiolabeled high molecular weight hyaluronate. As expected, extracts from detergent-solubilized membranes of encapsulated group A streptococcal strains (WF50 and WF51) and their streptomycin-resistant progeny (WF14 and WF13, respectively) were able to transfer glucuronic acid from UDP-glucuronic acid to high molecular weight polymer (Table 2; sp act 18,648; 21,910; 18,090; and 23,942 pmol/h/mg protein, respectively). In contrast, extracts obtained from the membranes of the transposon-inactivated mutants (WF15, WF20, WF22, and WF35) transferred only background amounts of glucuronic acid to high molecular weight polymer (Table 2; sp act 59, 136, 125, and 129 pmol/h/mg protein, respectively). Thus all of the acapsular transconjugants expressed <1% of the HAS activity relative to the parental strains.

Molecular Characterization of Acapsular Transconjugants. The

Table 1. *Bacterial Strains and Relevant Properties*

Strain	Description	Source
S. pyogenes		
WF50	Encapsulated M type 18 clinical isolate	J. Daly (4-50M)
WF51	Encapsulated M type 18 clinical isolate	J. Daly (ARF-8)
WF13	Encapsulated Str^r derivative of WF51	This paper
WF14	Encapsulated Str^r derivative of WF50	This paper
WF15	Acapsular transconjugant derived from WF13, multiple copies of Tn*916*	This paper
WF20	Acapsular transconjugant derived from WF13, multiple copies of Tn*916*	This paper
WF22	Acapsular transconjugant derived from WF14, multiple copies of Tn*916*	This paper
WF35	Acapsular transconjugant derived from WF14, multiple copies of Tn*916*	This paper
WF61	Acapsular transductant derived from WF20, ORFB::Tn*916* (contains deletion)	This paper
WF62	Acapsular transductant derived from WF22, ORFA::Tn*916*	This paper
WF63	Encapsulated revertant of WF62, Tc^r	This paper
WF64	Encapsulated revertant of WF62, Tc^s	This paper
E. coli		
CG120	DH1(pAM120), contains Tn*916*	D. Clewell
P2392	Host for recombinant λ phage	Stratagene Inc.
HB101	Host for cloning pACYC184 plasmids	Bethesda Research Laboratories
XL1-Blue	Host for cloning pBluescript plasmids	Stratagene Inc.
E. faecalis		
CG110	Donates Tn*916* at high frequency	D. Clewell

number of copies of Tn*916* in the acapsular mutants was determined by Southern blot analysis of whole-cell DNA using ^{32}P-labeled pAM120 (which contains a cloned copy of Tn*916*). Since a single restriction site for HindIII exists in the transposon, digestion generates left and right arms of 5.4 and 10.9 kb, respectively (33). Therefore, chromosomal DNA digested with HindIII will display two junction fragments for each copy of Tn*916*. No hybridization of the pAM120 probe was observed with WF51 DNA (Fig. 1 *A*, lane *1*), but was observed with a positive control (pAM120; Fig. 1 *A*, lane *5*). The WF20 transconjugant DNA (Fig. 1 *A*, lane *2*) possessed six hybridizing HindIII fragments (~17.5, 12.5, 11.5, 8.5, 6.3, and 5.8 kb), indicating that three copies of Tn*916* reside in the WF20 genome. The three other acapsular transconjugants also harbored multiple copies of Tn*916* (e.g., WF15, Fig. 1 *B*, lane *3*).

Transduction of Tn916-containing Loci into WF-50. To establish which Tn*916* insertion was responsible for loss of capsule production and HAS activity, generalized transduction was performed using the streptococcal bacteriophage A25. WF50 was infected with lysates of A25 phage (that had been previously grown on WF20), and plated on medium containing tetracycline to select for Tn*916*. Transductants appeared at a frequency of 1.5×10^{-7}/PFU phage, and both encapsulated and unencapsulated derivatives were recovered.

The exchange of single Tn*916* insertions into the wild-type background was then demonstrated by hybridization of transductant whole-cell DNA with the pAM120 probe (Fig. 1). Whole-cell DNA from a representative encapsulated transductant (Fig. 1 *A*, lane *3*) displayed hybridizing HindIII junction fragments of 12.5 and 5.8 kb, compared with the 17.5- and 8.5-kb HindIII fragments of the unencapsulated transductant WF61 (Fig. 1 *A*, lane *4*). Both pairs of hybridizing junction fragments were evident in the total-cell DNA of the WF20 transconjugant from which they were derived (Fig. 1 *A*, lane *2*). However, only the 17.5- and 8.5-kb pair of junction fragments cotransduced with the acapsular phenotype.

In a similar approach, a second acapsular mutant (designated WF62) was derived from the WF22 transconjugant by generalized transduction. WF62 whole-cell DNA contained a single copy of Tn*916* with hybridizing HindIII junc-

Table 2. *Hyaluronate Synthase Activity of Detergent Solubilized Streptococcal Membrane Preparations*

Strain	Encapsulation*	HAS
		(sp act‡)
WF50	+	18,648
WF51	+	21,910
WF13	+	23,942
WF14	+	18,090
WF15	−	59
WF20	−	136
WF22	−	125
WF35	−	129
WF61	−	156
WF62	−	0
WF63	+	12,345
WF64	+	18,400

* Encapsulation determined by India ink stain.
‡ Sp act; pmol glucoronic acid transferred from UDP-glucuronic acid/h/mg of protein.

tion fragments of 11.5 and 6.5 kb (Fig. 1 *B*, lane *2*). This hybridization pattern is distinct from that of WF61 DNA (Fig. 1 *B*, lane *1*). Thus, generalized transduction of the WF20 and WF22 transconjugants resulted in two classes of acapsular mutants, represented by the WF61 and WF62 transductants. Southern analyses indicated that an insertion similar to that found in the WF62 class of transductant was present among the multiple copies of Tn*916* in the WF15 and WF35 transconjugant DNA (data not shown).

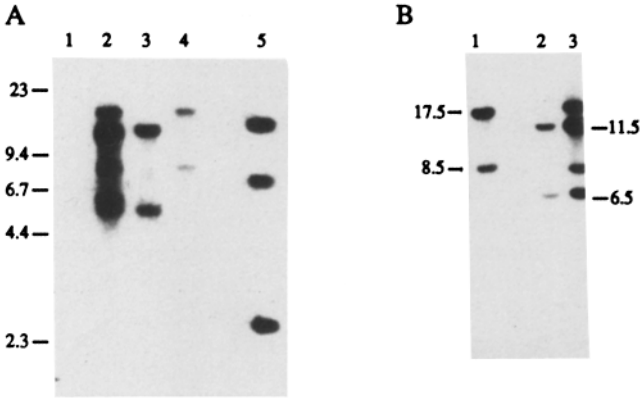

Figure 1. Southern hybridization of *S. pyogenes* DNA to identify Tn*916*-chromosome junction fragments. Whole-cell DNA was digested with HindIII and probed with ^{32}P-labeled pAM120 plasmid. (*A*) Lane *1*, WF51; lane *2*, WF20; lane *3*, a representative encapsulated transductant; lane *4*, WF61; lane *5*, EcoRI-HindIII-digested pAM120 (included as a positive control). Size standards (in kb) correspond to HindIII-digested λ DNA. (*B*) Whole-cell streptococcal DNA digested and probed as in *A*. Lane *1*, WF61; lane *2*, WF62; lane *3*, WF15. The sizes of hybridizing WF61 and WF62 junction fragments are indicated at the left and right, respectively.

After the Tn*916* insertions of the transductants were characterized, the HAS activity in the membranes of the two unencapsulated mutants was assayed. As seen in Table 2, both the WF61 and WF62 mutants lacked significant HAS activity (<1% relative to their respective parents). Mucoid transductants were not tested as they produced a demonstrable capsule. HAS assay of WF61 and WF62 confirmed, therefore, that the respective Tn*916* insertions were responsible for the loss of both capsule production and HAS activity.

Analysis and Cloning of the WF61 DNA Flanking Tn916. A probe for the DNA flanking Tn*916* in the WF61 class of acapsular transductants was generated by SSP-PCR amplification of group A streptococcal DNA. Unlike conventional PCR amplification of genomic DNA, SSP-PCR requires only one specific primer. The template for SSP-PCR is constructed by restricted digestion of genomic DNA and ligation to a known plasmid restriction fragment, followed by amplification in the presence of a second, plasmid-specific primer. Accordingly, the template for amplification of the WF61 left junction fragment was constructed by ligation of completely Sau3AI-digested streptococcal DNA to BamHI-linearized pUC18 plasmid DNA. The SSP-PCR reaction was then carried out using the TNL1 primer in conjunction with the pUC18-specific primer. Agarose gel electrophoresis of the reaction mixture revealed a PCR product of ~0.75 kb (Fig. 2, lane *2*). In addition to *S. pyogenes* DNA, this PCR product possessed the predicted pUC18 and Tn*916* sequences (data not shown).

To further confirm that the amplified DNA did indeed flank the transposon in WF61, the product from the above PCR reaction was cloned and used as probe in Southern hybridizations. The PCR product was digested with both TaqI and PstI and then ligated to ClaI-PstI–digested pBluescript. A 0.4-kb insert of amplified *S. pyogenes* DNA (and a small portion of the pUC18 polylinker) from one of the resultant chimeric plasmids (pGAC106) was then ^{32}P-labeled and used in Southern blot hybridizations. The pGAC106 probe hybridized to a 3.1-kb HindIII fragment of the wild-type WF50 DNA (Fig. 3 *A*, lane *1*), and the trans-

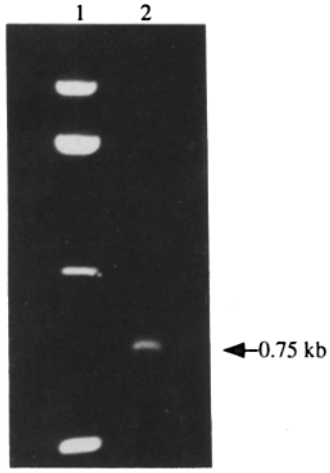

Figure 2. Amplification of *S. pyogenes* DNA by PCR. Whole-cell DNA from WF61 was amplified with the primers specific for the left terminus of Tn*916* (TNL1, 5′-TATAAAGCTTGAGAAGCAACAGGAGCGTCTTGTTGC-3′), and for pUC18 (M13 −47 primer, 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′). After 30 cycles of PCR, the reaction mixture was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Lane *1*, 1-kb ladder marker (Bethesda Research Laboratories); lane *2*, WF61 PCR reaction mixture.

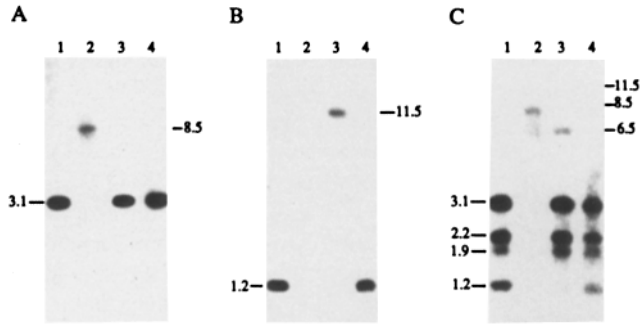

Figure 3. Southern hybridization of wild-type, transductant, and revertant group A streptococcal DNA. Whole-cell DNA was digested with HindIII and probed with (*A*) pGAC106, (*B*) pGAC126, and (*C*) pGAC130 DNA. Lane *1*, WF51; lane *2*, WF61; lane *3*, WF62; lane *4*, WF63. The 11.5-kb band in *C*, lane *3* was visible upon prolonged exposure.

ductant WF62 DNA (Fig. 3 *A*, lane *3*). Hybridization of the pGAC106 probe to WF61 DNA, however, resulted in an 8.5-kb HindIII fragment (Fig. 3 *A*, lane *2*). This hybridizing restriction fragment was approximately 5.4 kb larger than the wild-type fragment, indicating that the left terminus of Tn*916* inserted ~3 kb from a HindIII site in the WF61 genome (see Fig. 4). The 8.5-kb fragment was also demonstrated in WF61 DNA when the transposon was used as a probe (e.g., Fig. 1 *B*, lane *1*), confirming that it was indeed the left HindIII junction fragment.

The pGAC106 insert was then employed to probe a λ replacement vector library of WF14 DNA and identify a nonmutagenized copy of the DNA involved in capsule production. Positive plaques were obtained at a frequency of ~1

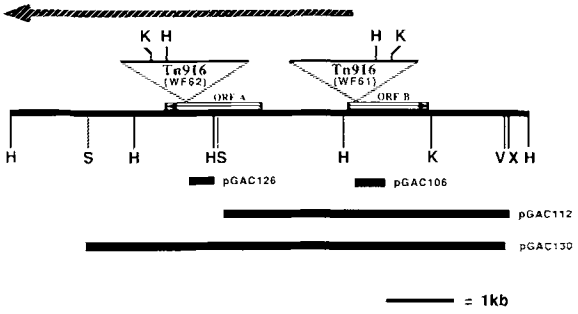

Figure 4. Restriction map of wild-type DNA for the locus involved in capsule production. (——) Orientation of the respective Tn*916* insertions of WF61 and WF62 (~1/8 scale). (-----) Position of the WF61 deletion extends approximately 2 kb beyond the chromosomal DNA illustrated here. In WF61 and WF62, the transposon inserted into two open reading frames, ORFA and ORFB (▸ designating putative direction of transcription). Restriction sites are abbreviated as follows: H = HindIII, K = KpnI, S = Sau3AI, V = PvuII, and X = XbaI. For clarity, the only chromosomal Sau3AI sites represented (*hatched bars*) are those cloned into the λ replacement vector polylinker in the construction of λBD5 and λBD21. DNA subcloned into pACYC184 from λBD5 and λBD21 is indicated by the solid bars labeled pGAC112 and pGAC130, respectively. The DNA flanking the Tn*916* insertions of WF61 and WF62 was amplified by PCR and cloned into pBluescript to create the pGAC106 and pGAC126 plasmids, respectively.

in 200. One positive clone (λBD5) was isolated and its DNA further analyzed. SalI digestion of the λBD5 DNA resulted in the expected λ DNA restriction fragments, as well as a fragment of 12 kb, which corresponds to the entire streptococcal DNA insert. Digestion of this insert with XbaI yielded three restriction fragments of 5.2, 4.5, and 2.2 kb. The 4.5-kb XbaI fragment hybridized with the pGAC106 probe and was therefore cloned into the pACYC184 plasmid using SalI and XbaI. The pACYC184 vector was chosen to bypass potential stability problems of this group A streptococcal insert in *E. coli*, as was reported by Kehoe and Timmis (34). Antibiotic sensitivity and DNA hybridization indicated that one recombinant plasmid, pGAC112, contained the proper insert.

Further analysis of pGAC112 indicated that it contained internal HindIII and KpnI restriction sites separated by 1.4 kb. The pGAC106 probe hybridized to a region proximal to this internal HindIII site, suggesting that Tn*916* inserted extremely close to a HindIII site in WF61. Restriction mapping and DNA sequence analysis of pGAC112 and WF61 has identified the insertion site of the transposon in WF61. This site is situated 125 bp from the HindIII site of wild-type DNA, and is illustrated in Fig. 4. Subsequent DNA hybridization analyses have demonstrated, however, that genomic DNA has been deleted from this insertion site to a region 6–8 kb distal from the right terminus of Tn*916* in WF61 (see below).

Analysis and Cloning of the DNA Flanking the Tn916 Insertion of WF62. A probe was also generated for the WF62 class of transductants by SSP-PCR amplification of DNA sequences adjacent to the transposon. A template of HindIII-digested WF62 DNA ligated to similarly digested pUC18 DNA was amplified in the presence of the primers specific for Tn*916* (TNR1) and pUC18 DNA. This PCR reaction resulted in a 0.7-kb product consisting of amplified *S. pyogenes*, pUC18, and Tn*916* (data not shown).

To clone the right Tn*916*-chromosomal junction fragment of WF62, the PCR product was digested with HindIII, then ligated to HindIII-linearized pBluescript to create pGAC126. ^{32}P-labeled pGAC126 insert was then used to probe wild-type and mutant whole-cell DNA. The pGAC126 DNA hybridized to a 1.2-kb HindIII fragment of wild-type DNA (WF51; Fig. 3 *B*, lane *1*) and to an 11.5-kb HindIII fragment of WF62 DNA (Fig. 3 *B*, lane *3*). The latter band corresponds to the right Tn*916* HindIII junction fragment of WF62 (see Fig. 1 *B*, lane *3*, and Fig. 4). Surprisingly, the WF61 DNA did not hybridize with the pGAC126 probe (Fig. 3 *B*, lane *2*), even upon prolonged exposure.

The pGAC126 probe was then used to identify a nonmutagenized copy of the DNA flanking the WF62 transposon insertion. Southern hybridizations were first performed with λBD5 DNA in an effort to determine if DNA homologous to the pGAC126 probe resided in the λBD5 insert. The pGAC126 probe did not hybridize with λBD5 DNA, demonstrating that the DNA inactivated by the transposon in WF62 was not yet cloned. Therefore, radiolabeled pGAC126 insert DNA was used to screen the λ library of WF14 DNA. One positive clone (λBD21) possessed 2 kb of DNA not in-

cluded in λBD5. DNA from λBD21 was purified, digested with XbaI and PvuII, and cloned into XbaI-EcoRV–digested pACYC184 to create pGAC130 (see Fig. 4). Further analysis indicated that in WF62, Tn*916* inserted ~2.6 kb away from the transposon insertion site of WF61. This insertion apparently inactivated the gene product of an open reading frame (ORFA) which is oriented divergently from the open reading frame mutated in WF61, and ORFB (see Fig. 4).

Demonstration of a Deletion in WF61. The failure of WF61 to hybridize with the pGAC106 probe (Fig. 3 *B*, lane *2*) leads us to hypothesize that a deletion of genomic DNA had occurred. Further evidence for this deletion included the presence of an unpredicted WF61 HindIII right junction fragment (a 17.5-kb band [Fig. 1 *A*, lane *4*] is present instead of a predicted 11-kb band), as well as the amplification of a WF61 right junction PCR product containing DNA that did not correspond to the expected product (data not shown). Therefore the pGAC130 insert was used as a probe in DNA blots to estimate the extent of DNA deletion. As expected, the pGAC130 insert hybridized to wild-type HindIII fragments of 3.1, 2.2, 1.9, and 1.2 kb (Fig. 3 *C*, lane *1*). Hybridization of this probe to WF62 DNA results in hybridization of all but the 1.2-kb HindIII fragment, now replaced by the chromosome-transposon junction fragments of 11.5 and 6.5 kb (Fig. 3 *C*, lane *3*; note that the 11.5-kb fragment was visible after prolonged exposure). The only hybridization of pGAC130 with WF61 DNA is the 8.5-kb left junction fragment (Fig. 3 *C*, lane *2*), indicating that at least 5.6 kb of DNA beyond the right Tn*916* terminus has been deleted from the genome (see Fig. 4).

Reversion of Mutants to Capsule Production. Revertants of Tn*916*-mutagenized group A streptococci have been isolated in the absence of selective pressure for the transposon (32). Accordingly, WF62 was passaged in media lacking tetracycline and plates were screened for mucoid colonies. Plating of the WF62 stock directly onto media lacking the antibiotic failed to result in encapsulated colonies (~30,000 colonies were examined). An overnight passage of the acapsular WF62 mutant in liquid media was sufficient to recover roughly 1 in 1,000 capsule-producing streptococci. Both Tc^s and Tc^r revertants appeared, with the former being more frequently obtained ~83% of the time.

After isolation of encapsulated revertants, Southern analyses were performed to determine if the transposon had excised from ORFA. Whole-cell DNA was isolated from one Tc^r revertant (WF63) and hybridized with ^{32}P-labeled pGAC126 insert. As seen in Fig. 3 *B*, WF63 now possesses the 1.2-kb HindIII fragment (lane *4*). A wild-type pattern was also observed upon hybridization with pGAC130 (Fig. 3 *C*, lane *4*), and pGAC106 (Fig. 3 *A*, lane *4*). DNA from a representative Tc^s revertant (WF64) hybridized in a similar fashion with ORFA-specific probes (data not shown). Assay of WF63 and WF64 membranes indicated that HAS activity was restored (Table 2; sp act 12,345 and 18,400, respectively). Therefore, the encapsulated variants of WF62 examined no longer possessed a Tn*916* insertion in ORFA and demonstrated HAS activity approaching that of the encapsulated group A streptococci.

Discussion

In addition to the numerous studies that correlate hyaluronate capsule production with virulence of group A streptococci, the recovery of highly mucoid isolates from recent outbreaks of acute rheumatic fever emphasizes the need to characterize the production of this polysaccharide. Using Tn*916* mutagenesis, nonmucoid transconjugant colonies were derived from mucoid strains of group A streptococci, and were shown to lack demonstrable capsule. None of the mutations characterized in this study affected the genes required for the production of the nucleotide-sugar precursors of hyaluronic acid. Since precursor nucleotide sugars are part of the HAS assay reaction mixture, membranes from a mutant of this class still should be able to produce hyaluronic acid. The background levels of radiolabeled substrate incorporation indicated that all acapsular transconjugants possessed <1% of the HAS activity of encapsulated isolates.

Because of the presence of multiple copies of the transposon in transconjugants, however, generalized transduction was required to establish linkage between the mutant phenotype and a single Tn*916* lesion. Two distinct classes of acapsular, HAS^- transductants resulted from these marker exchange studies, and further molecular analyses of the Tn*916*-inactivated DNA of WF61 and WF62 defined a locus required for HAS activity. Finally, the isolation of encapsulated WF62 variants with HAS activity approaching that of wild-type confirmed that the Tn*916* insertion into ORFA was responsible for the loss of hyaluronate biosynthesis. The WF63 revertant apparently possessed two-thirds the HAS activity of its streptomycin-resistant parent, while the enzyme activity of WF64 was equivalent to that of WF14. One possible explanation for the decrease in WF63 HAS activity is alteration of the ORFA sequence and thus the ORFA gene product. In any case, these experiments firmly established the requirement for an intact ORFA in capsular hyaluronate biosynthesis.

This is the first report, of which we are aware, that demonstrates such a large deletion of host DNA accompanying a Tn*916* insertion. The transposon appears to be intact based on restriction analyses and the sequences of Tn*916* in the PCR products. Based upon restriction hybridization data of WF61 versus wild-type DNA, the deletion is estimated to be ~6–8 kb. The transconjugant WF20 also harbored this deletion, which suggests that it did not occur during the transduction procedure. Our application of SSP-PCR to Tn*916*-mutagenesis studies provided a fast, convenient method for detecting the deletion. This technique will allow other researchers using Tn*916* to rapidly generate probes for the inactivated DNA and sequence transposon-chromosome junction fragments.

Although transposon mutagenesis implicates a role for ORFA in HAS activity, one cannot exclude the possibility that the HAS^- phenotype of WF62 results from the interruption of transcription of downstream genes. The deletion that accompanied the Tn*916* insertion of WF61 included the entire ORFA. It is probable that this deletion, rather than the disruption of ORFB, is responsible for the loss of HAS activity. It is also possible that other genes required for HAS activity were lost in WF61. However, the deletion may pro-

vide a unique opportunity for complementation studies using the WF61 mutant. By reintroduction of various portions of the DNA lost in this transductant, enzyme activity should be fully restored upon addition of the proper combination of HAS genes. Experiments are currently in progress to address these points. Another approach to demonstrate HAS activity involves expression of cloned genes in bacteria other than group A streptococci. Although most strains of *E. coli* do not synthesize the UDP-glucuronic acid precursor, an exogenous source of this substrate is provided in the HAS assay. Preliminary studies with detergent-solubilized *E. coli* harboring pGAC130 suggest that we have yet to reconstitute enzyme activity. Besides the need to optimize the assay for other bacteria, full expression of enzyme activity in heterologous hosts may require other HAS components and/or streptococcal regulatory factors not present in the cloned DNA sequences.

It has been suggested that the *virR* gene (35) regulates hyaluronate biosynthesis. Deletions in this gene, located upstream of the M protein and C5a peptidase structural genes, result in decreased expression of these two surface-associated virulence factors. It was also demonstrated that variants harboring deletions of *virR* no longer expressed capsule. Preliminary Southern hybridization studies performed in our laboratory indicate that *virR* is not closely linked to the locus required for HAS activity. Isolation and characterization of a larger sample of acapsular mutants should yield transposon insertions in *virR* if this gene truly regulates capsule production. In addition, quantitation of the levels of HAS-specific mRNA and HAS enzyme in *virR* mutants may provide more evidence for regulation of hyaluronate synthesis by this gene.

An acapsular mutant (TX4) similar to the WF61 transductant was recently derived from an M type 18 group A streptococcal strain by Wessels et al. (2). DNA hybridization and sequence analysis performed by our laboratory indicates that WF61 and TX4 harbor the same deletion/insertion. Insertion into the same site can be explained by a preferred Tn*916* target sequence in the genome of these *S. pyogenes* strains. However, the presence of the same deletion in these two independently isolated transductants is intriguing. It is possible that factors produced by the transposon somehow interacted with sequences in the host genome and induced the deletion of intervening DNA. Another explanation involves recombination between the transposon in ORFB and a second copy of Tn*916* situated 6–8 kb away.

Liquid passage of WF61 has so far failed to result in encapsulated revertants, and acapsular mutants possessing this deletion/insertion are presumably nonrevertable. It must be confirmed, however, that the deletion of such a significant portion of the genome has not affected other streptococcal factors. Passage of WF62 in the absence of tetracycline resulted in Tc^s- and Tc^r-encapsulated revertants. *S. pyogenes* variants, both sensitive and resistant to the antibiotic, were also isolated by Nida and Cleary (32). WF62 is also an important mutant for studies of hyaluronate synthesis since the Tn*916* insertion has not caused any detectable change in nearby DNA and includes the DNA deleted in WF61. However, an ideal isogenic mutant for future studies should be constructed by, for example, allelic replacement of genes required for capsule biosynthesis. On the basis of transposon mutagenesis, ORFA has been identified as an HAS gene in this study. Further analysis should determine which other regions of the group A streptococcal genome are required for hyaluronate synthesis.

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Address correspondence to Dr. Ivo van de Rijn, Wake Forest University Medical Center, Medical Center Blvd., Winston-Salem, NC 27157.

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