Isolation of a *Streptococcus pyogenes* Gene Locus That Directs Hyaluronan Biosynthesis in Acapsular Mutants and in Heterologous Bacteria*

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A contiguous 3-kilobase pair region of DNA was isolated from Group A *Streptococcus pyogenes* (GAS) that can direct hyaluronic acid (HA) capsule biosynthesis in acapsular mutants as well as heterologous bacteria. The DNA was identified by transposon 916 insertional mutagenesis and subcloned into a plasmid shuttle vector. Mutant acapsular GAS or *Enterococcus faecalis* containing this plasmid, but not vector alone, displayed a mucoid phenotype on agar plates, possessed a capsule as seen by light microscopy, and produced HA in quantities comparable with wild-type GAS. The polysaccharide was shown to be authentic HA based on its recognition by a specific proteoglycan and its degradation by *Streptomyces* hyaluronate lyase. *Escherichia coli* with the complementing plasmid also produced HA but at only 10% of the level made by the above cells. *E. coli* minicell analysis showed that two proteins, 42 and 45 kDa, are expressed by the functional DNA insert. Deletion analysis of the insert in the minicells revealed that the 42-kDa protein is essential for HA production. This is the first demonstration of reconstitution of HA capsule biosynthesis in *vivo*.

Group A *Streptococci* (GAS) are responsible for numerous diseases of man such as pharyngitis, erysipelas, and rheumatic fever. After a relative absence of serious disease, a recent resurgence of virulent strains has been noted, but its basis has not been elucidated (1–4). One widespread strategy utilized by bacterial pathogens to evade host defenses is capsule production; the extracellular polymer coating physically hinders phagocytosis and complement-mediated lysis (5). GAS produce a capsule composed of HA, a polysaccharide consisting of alternating gluconic acid and N-acetylglucosamine residues. Recently, the HA capsule was confirmed to be a virulence factor that protects GAS from *in vitro* phagocytosis and killing (6). Encapsulated GAS were 100-fold more virulent in *vivo* in mice than the corresponding Tn-induced isogenic acapsular mutants (6). HA is found in the extracellular matrix of all vertebrate animals as well as in the Group A and Group C streptococcal capsules; the host is therefore faced with the dilemma of mounting an immune response to self, due to molecular mimicry (7). The molecular details of HA synthesis are not yet clear in either prokaryotic or eukaryotic organisms, since the enzyme that polymerizes the polysaccharide, HA synthase, has not been purified or cloned from either source. Here we report that a locus encoding at least two streptococcal proteins can direct HA production in acapsular mutant GAS as well as in *Enterococcus faecalis* and *Escherichia coli*.

**EXPERIMENTAL PROCEDURES**

**Materials—**Media reagents were from Difco. Restriction and DNA modifying enzymes were from Prokine unless otherwise noted. Synthetic oligonucleotides were made at the University of Texas Medical Branch Synthesis Facility or by Keystone Laboratories. All other reagents were of the highest grade available from Sigma except where stated otherwise.

**Strains and Vectors—** *E. coli* was maintained on Luria broth and grown in Superbroth with antibiotics for plasmid production. Other bacteria were grown as standing cultures on Todd-Hewitt broth supplemented with 1% yeast (THY) and horse serum (5–10%, Life Technologies Inc.). Cultures to be assayed for HA were grown using the dialysate from dialyzed THY broth (i.e. nutrients <10–14 kDa). The mucoid GAS strain, S43/19294, was obtained from the Rockefeller Collection (8). Spontaneous strep* strain used as Tn acceptors were selected by plating ~10⁸ cells on THY plates containing 1 µg/ml streptomycin and 5% defibrinated sheep blood (Colorado Serum). E. faecalis CG110, a Tn916 donor (tetracycline-resistant, 5 µg/ml), and pAM118, a plasmid with a Tn916 insert, were generously supplied by D. Clewell (9). The *E. coli*/ *Gram-positive shuttle vector pAT19 (erythromycin-resistant, 8 µg/ml) for Gram-positive or -negative strains was obtained from P. Courvalin (10). The *E. faecalis* host strain CG110 was obtained from G. Dunny (11). *E. coli* minicell strain Y1448 (12) was supplied by R. Macnab. The *E. coli* hosts used were SURE, XLI-Blue (Stratagene), LES32, and KW251 (Promega).

**DNA Purification and Sequencing—**Streptococcal chromosomal DNA was obtained by the method of Caparon and Scott (13). *E. coli* plasmid DNA was purified by the Isogentrap method (5 Prime–3 Prime, Inc.) for screening or by the SDS/alkali method for cloning and blotting procedures (14). Agarose (Bio-Rad) gel-isolated DNA (<7 kb) was purified by GeneClean (Bio 101), while longer fragments were isolated using GlassMax cartridges (Life Technologies Inc.) to minimize shearing. λ DNA was prepared from phage purified on glycerol gradients (14). Sequencing of double-stranded plasmids was performed with Sequenase 2.0 (U. S. Biochemical Corp.) and α-[32P]thiodATP (Amersham Corp.).

**Library Production—**The AZAPII library (Stratagene, 1–10 kb capacity) was converted to plasmid DNA digested extensively with EcoRI. The AGEM system inserts (Promega, 9–23 kb capacity) consisted of 543 DNA partially digested with Sau3A1. The AZAP system inserts from selected plasmids were excised and converted to plasmid form by coinfection with M13 helper plasmid (R408 or Exassist) according to Stratagene protocols.

**HA Synthase Preparation and Assay—**HA synthase was obtained from membranes of late log phase cells disrupted by sonication in PBS (20% cell suspension, dry ice/50% methanol bath, 4 × 2 min, Heat Systems W–380 with microprobe). Debris was removed by centrifugation at 12,000  ×  g for 10 min at 4 °C, and then the membrane fraction was suspended from the supernatant by ultracentrifugation (100,000  ×  g for 60 min). The membranes (5–300 µg of protein) were incubated with UDP[14C]glucuronic acid (250 mCi/mmol, ICN) in the assay as described by Tricott and van de Rijn (15). Specificity of polymerization was tested by omitting UDP-GlcNAc. Incorporation of the radiolabel

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‡ The abbreviations used are: GAS or GCS, Group A or Group C *Streptococci*; HA, hyaluronic acid or hyaluronan; THY, Todd Hewitt/Yeast; dTHY, dialyzed from THY; Tn, transposon; PBS, phosphate-buffered saline; C7AB, cetyltrimethylammonium bromide; kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis.
into high M, polymers was measured by paper chromatography (16). *Transposon Mutagenesis and Mutant Selection—The detailed methods for Tn mutagenesis, mutant selection, and the isolation and characterization of the Tn-tagged DNA will be described elsewhere. 2 Briefly, Tn insertional mutagenesis was done on a mucoid strep' S43 strain using the method of O'Connor and Cleary (17). The nonmucoid mutant colony was enriched by Percoll step gradients in analogy to work done for Tn mutagenesis, mutant selection, and the isolation and characterization of extracellular HA synthesized by a sensitive HA assay (see "HA Polysaccharide Analysis"). Transduction with the streptococcal phage A25 (kindly supplied by M. Caparon) was used to determine the relevance of the various Tn916 insertions in the nonmucoid strains (13). *Transposon Mapping and Isolation of the HA Synthesis Locus—An overview of the isolation procedures, to be described elsewhere, follows. After electrophoresis of chromosomal HindIII digests, the agarose gels (0.5–0.6%) were dried down directly (20) and probed with the Tn916-containing EcoRI fragment of pAM18 labeled by the random primer method. It was noted that one of the Tn916/S43 chimeric DNA fragments consistently migrated slower than the other fragments in HindIII digests of wild-type DNA. This chimeric fragment from preparative gels (5–15 µg) was isolated from an agarose gel with GlassMax and used as a DNA sequencing template to determine the sequence of the Tn916/S43 insertion; this DNA was not cloned. A synthetic oligonucleotide (TGGCACAATATGT-CAGCCC) derived from the termini of the right HindIII fragment of Tn916 (21) that reads outward from the Tn (AAAGTGT-GATAAGTCC) was employed as a primer in a modification of the Sequenase method (U. S. Biochemical Corp.) for plasmids. The intact wild-type DNA was then obtained by screening the λ libraries in the typical fashion (14) with end-labeled oligonucleotide (TGGCACAATATGTCAGCC) derived from the chromosomal sequence determined as above. *HA Polysaccharide Analysis—The presence of HA in bacterial cultures (early log for S43 derivatives, late log for all others) was determined using the HA TEST radiometric assay (range, 0.01–104 cpm, Pharmacia LKB Biotechnology Inc.). The detection is based on inhibition of 125I-proteoglycan binding to HA immobilized on beads by soluble HA in the sample. Secreted or released HA in cultures grown on dTHY was measured by assay of the supernatant fraction after centrifugation (11,000 g for 5 min). Cell-associated HA was determined by extracting the cell pellet in 0.1 volume of PBS containing 0.01% SDS for 40 min at 37 °C. The cells were then removed by centrifugation as above. The final SDS concentration in the HA assay never exceeded 0.001%. HA was also purified by CTAB precipitation. The pooled medium supernatant and cell extracts (treated with 0.1 mg/ml trypsin for 40 min at 37 °C) were then extracted with 25-ml chloroform, the pH was adjusted to 0.3% CTAB and allowed to stand at 37 °C for 15 min. The precipitate was collected by centrifugation (3,000 x g for 20 min at room temperature) and resuspended in 0.7 ml of 2 M NaCl with gentle mixing for 40 min. The solubilized fraction (clarified by high speed centrifugation, 11,000 x g for 5 min) was then precipitated by addition of 2 volumes of ethanol. After 5 min, the solids were collected by high speed centrifugation. The pellet was washed with 70% ethanol, 30% 2 M NaCl and then 70% ethanol. After brief drying, the pellet was resuspended in 2 ml NaCl, and ethanol precipitation was repeated as above. The final pellet was dissolved in 0.2 ml of water overnight with gentle mixing at 4 °C. The uronic acid content of the purified material was measured by the carbazole assay with glucuronic acid as standard (22). The M, of the polymers and authentic rooster comb HA standards (Lifecore) were compared by PAGE (23) and size exclusion chromatography. To verify the nature of the polysaccharide, samples were digested with hyaluronidase from Streptococcus hyaluronidicus in 50 mM sodium acetate, pH 5.3, at 42 °C before analysis. Sepharose 4B (Pharmacia, 1 x 25-cm column, 20-ml bed volume) eluted with PBS was used to fractionate the various polymers. The column fractions were assayed by the carbazole method, and the HA test was used to confirm the major peak identity. The column was calibrated with dextran (2 x 106, 5 x 105, 4 x 104 Da, Pharmacia) and lactose as well as the HA standards. *Minicell Analysis—The identity of plasmid-encoded polypeptides was determined by radiolabeling proteins produced in minicells (24). Minicells from E. colit 1448 containing pAT19, pPD41Δ5, or pPD41Δ7 were harvested from sucrose gradients and washed with PBS containing 0.01% gelatin. The minicells were incubated at 37 °C for 1 h in minimal salts with glyceral and all amino acids except Met and Cys. The minicells were then labeled with Tran35S-label (ICN) for 30 min at 37 °C followed by a 5-min Met/Cys chase (25). The minicells were then washed with PBS and analyzed by SDS-PAGE after boiling (3 min) in Laemmli sample buffer (25). The gels were stained with Coomassie Blue, dried, and exposed to XAR-5 film (Kodak). *Miscellaneous—-of several published electroporation methods for use with Gram-positive bacteria, we found that only the technique of Caparon and Scott (13) was successful in transforming S43 derivatives with plasmids (0.5–20 colony-forming units/µg DNA). To create a deletion set, the pPD41 plasmid linearized with XbaI and SphI was truncated by limited exonuclease III digestion and mung bean nuclease treatment according to the Stratagene kit. The ligation mixtures were transformed into Epicurean SURE cells (Stratagene) and screened for insert size. E. coli was also transformed by the Ca2+ method (14). *RESULTS AND DISCUSSION Tn insertional mutagenesis was used to tag and to mutate capsule biosynthesis genes of GAS. The bacteriophage A25-transducing lysate from one acapsular mutant, designated S43Tn7 and containing two Tn916 elements, was found to transmit the nonmucoid phenotype to 3 out of 5 transductants. 2 The two Tn elements were segregated by transduction; one Tn insertion characterized by higher M, HindIII fragments was found only in the nonmucoid transductants (S43TdT7N), while the other Tn insertion event producing lower M, fragments was found only in the mucoid cells (S43TdT7M). 2 Nonmucoid transductants did not possess HA synthase activity or a capsule by microscopy. The gel-purified, Tn-tagged chromosomal DNA from S43Tn7 was directly used as a template in sequencing reactions with a Tn-specific primer that reads outward from the Tn terminus and into the insertion site. An oligonucleotide probe corresponding to a portion of the sequence of the interrupted DNA associated with the nonmucoid phenotype was then used as a hybridization probe for screening wild-type S43 genomic DNA libraries in A phage. An excised λZAP clone, designated pB3, containing a 5.5-kb EcoRI fragment was obtained (Fig. 1). However, differences between the wild-type and Tn mutant genomes were noted by Southern analysis. 2 Since previous studies showed that Tn916 can cause deletions of chromosomal DNA (26), we then determined that at least 4 kb of DNA flanking the small arm (5-kb HindIII portion of Tn) was missing in S43Tn7 and S43TdT7N (Fig. 1). Therefore, a larger wild-type genomic fragment spanning the deletion was obtained from the λGEM library. A 6.6-kb portion of DNA adjacent to pB3 was

![Fig. 1. Restriction map of the streptococcal HA biosynthesis locus. EcoRI (E) restriction sites on the wild-type S43 chromosome are illustrated with respect to the relevant Tn insertion and the minimal extent of the associated deletion (boxed box). The region of DNA capable of directing HA synthesis in mutants and heterologous species, pPD41Δ5, is shown in detail below. Two HindIII (H), two PstI (P), and one BglII (B) sites were found. KpnI, BamHII, SalI, SacI, Smal, Spal, and XbaI did not cut the pPD41Δ5 insert. The multiple cloning site (M) is the result of fusion of the deleted, blunt ended DNA and the pAT19 M. The inserts of the initial clones pB3 and pPD41 are shown above the genomic map. The large EcoRI fragment on the extreme left is ~20 kb and not shown to scale.](image-url)
subcloned into pAT19 and designated pPD41 (Fig. 1).

When pPD41 was electroporated into the original acapsular Tn mutant, S43Tn7, or a spontaneously arising nonmucoid strain, S43Tn11, transformant colonies displayed the mucoid phenotype on agar plates, while cells with pB3 or pAT19 were nonmucoid (not shown). The capsules of the complemented cells were easily visualized by microscopy with India ink and were indistinguishable from the wild type (Fig. 2). Ovine testicular hyaluronidase treatment of the cultures completely destroyed these capsules. Using a sensitive radiometric assay, HA was detected in the cultures of the Tn mutants containing pPD41 in amounts comparable with the wild-type parent (Table I). Transformants with pB3 or pAT19, as well as the original mutant without plasmid, did not produce HA (Table I). The HA was detected by proteoglycan binding; this high affinity interaction is very specific and is widely accepted as evidence for the presence of HA (27). As in the case of the GAS mutants, E. faecalis or E. coli containing pPD41 produced HA (Table I). By microscopy with India ink, E. faecalis, but not E. coli, containing pPD41 possessed a substantial capsule (not shown).

To determine the minimum size of the locus directing HA biosynthesis, the complementing DNA insert of pPD41 was reduced by limited exonuclease digestion of the plasmid from apparent "background" biosynthesis, the complementing DNA insert of pPD41 was reduced by limited exonuclease digestion of the plasmid from apparent "background" biosynthesis, the complementing DNA insert of pPD41 was reduced by limited exonuclease digestion of the plasmid from apparent "background" biosynthesis, the complementing DNA insert of pPD41 was reduced by limited exonuclease digestion of the plasmid from apparent "background" biosynthesis, the complementing DNA insert of pPD41 was reduced by limited exonuclease digestion of the plasmid from apparent "background" biosynthesis. As in the case of the GAS mutants, E. faecalis or E. coli containing pPD41 produced HA, while cells formed with plasmids containing an insert of -3 kb (e.g. 5-min deletion, pPD41A5; see Fig. 1) still produced HA, while cells with smaller inserts (e.g. 6-min deletion, pPD41A6, -2.3 kb or 7-min deletion, pPD41A7, -1.7 kb) did not make HA (Table I). The E. faecalis cells transformed with pPD41A5 produced hyaluronidase-sensitive capsules as assessed by microscopy (Fig. 2, D and F) and formed mucoid colonies on agar plates, whereas the cells containing pPD41A7 were equivalent to untransformed E. faecalis (Fig. 2E). E. faecalis has not been reported to produce a capsule or any exopolysaccharides. Therefore, the pPD41A5 insert is responsible for HA capsule biosynthesis.

The extracellular polysaccharides produced by the various bacteria containing the pPD41 family of plasmids were further characterized by gel filtration chromatography and PAGE. All polysaccharides possessed $M_r$ values on the order of $10^6$, since they eluted in the void volume and the first included fractions were electrophoresed on a 4% gel and stained with Alcian Blue (23). One pg (by carbazole assay) samples were loaded in lanes A–H, and 8-μg samples were loaded in lanes I–K. Lanes A: S43, B, S43Tn7(pAT19), C, S43Tn7(pPD41); D, E. faecalis (pPD41A5); E, E. faecalis (pPD41A7); F, E. faecalis (pPD41A5) treated with 500 units/ml hyaluronidase (type V, 2000 units/mg) for 30 min at 37°C.

![Fig. 2. Visualization of HA capsules in transformed bacteria by light microscopy. These photomicrographs of early log cultures stained with India ink (19) were taken on a Leitz Laborlux microscope at x1000 magnification. The results depict the ability of plasmids pPD41 or pPD41A5, but not pAT19 alone or pPD41A7, to direct HA capsule biosynthesis after transformation into the acapsular Streptococcus pyogenes mutant S43Tn7 or into normally acapsular E. faecalis. The bright halo surrounding the cells is the HA capsule. Ovine testicular hyaluronidase treatment destroyed the capsule. Panels: A, wild-type S43; B, S43Tn7(pAT19); C, S43Tn7(pPD41); D, E. faecalis (pPD41A5); E, E. faecalis (pPD41A7); F, E. faecalis (pPD41A5) treated with 500 units/ml hyaluronidase (type V, 2000 units/mg) for 30 min at 37°C.](image)

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<th>TABLE I</th>
<th>HA production by various constructs</th>
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<td>Bacterial strain</td>
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a The media alone contains about 1 ng/μl HA after dialysis. The apparent "background" is reported for each host.

b S43Tn11 is an acapsular, HA synthesize-negative, Tn-containing strain that was shown by transduction analysis to be a spontaneous mutant.

![Fig. 3. PAGE analysis of polysaccharides produced by various transformed bacteria. Authentic HA (Std) and polysaccharides purified from cell cultures as described under "Experimental Procedures" were electrophoresed on a 4% gel and stained with Alcian Blue (23). Strains without pPD41 or pPD41A5 do not produce HA. The majority of the polymer population in each sample migrated similarly to high M₉ HA (lanes F and G). The bracket on the right marks the extent of staining of the low M₉ HA standard, which did not photograph well (lane H). The arrowhead indicates the top of the gel. Streptomyces hyaluronate lyase (HAase) treatment (20 units, 15 min) completely degraded the bacterial products. One-μg (by carbazole assay) samples were loaded in lanes A–H, and 8-μg samples were loaded in lanes I–K. Lanes A: S43, B, S43Tn7(pAT19); C, S43Tn7(pPD41); D, E. faecalis (pPD41A7); E, E. faecalis (pPD41A5); F, native HA, viscosity = 13,172; G, HA with viscosity = 1,589, H, HA with viscosity = 20,1, hyaluronate lyase-treated truncated sample C; J, hyaluronate lyase-treated sample E; K, hyaluronate lyase-treated sample F.](image)
containing the complementing plasmids (Fig. 3).

The E. coli minicell system provides a convenient way to determine the number and size of proteins encoded by genes on episomal plasmids (12, 24). Minicell analysis revealed that at least two proteins were encoded on the complementing DNA 45-kDa species, indicating that the former protein is essential for HA synthesis. We calculate that about 80% of the coding capacity of the ~3-kb insert in pPD41A5 is utilized for these 45-kDa proteins and their specific roles in HA capsule biosynthesis.

Note Added in Proof—Dougherty and van de Rijn (Dougherty, B. A., and van de Rijn, I. (1993) J. Biol. Chem. 268, 7118–7124) recently reported the sequence of the GAS gene for UDP-Glc dehydrogenase. We have independently sequenced the gene coding for the 45-kDa protein in the x1448(pPD41A5) minicells; it is the GAS dehydrogenase gene (99.8% identity). The 42-kDa gene product can direct HA biosynthesis in the absence of this functional GAS dehydrogenase and is, therefore, the GAS HA synthase gene hasA.

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REFERENCES


FIG. 4. SDS-PAGE analysis of proteins synthesized by pPD41 deletion plasmids in E. coli minicells. Minicells labeled with [35S]Met/Cys were lysed by boiling in SDS-sample buffer and electrophoresed on a 10% gel. Cells containing the pPD41A5 plasmid produce HA, and two proteins are seen on this autoradiogram (24-h exposure) at 42 and 45 kDa (lane 1, positions marked with arrows) that are not produced by vector alone (lane 3). Cells containing the pPD41A5 plasmid do not produce HA and only synthesize the 45-kDa protein (lane 2). Standards (Bio-Rad, Low M), are shown in kDa.