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Molecular Analysis of the Capsule Gene Region of Group A *Streptococcus*: the *hasAB* Genes Are Sufficient for Capsule Expression

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Enzymes directing the biosynthesis of the group A streptococcal hyaluronic acid capsule are encoded in the *hasABC* gene cluster. Inactivation of *hasC*, encoding UDP-glucose pyrophosphorylase in the heavily encapsulated group A streptococcal strain 87-282, had no effect on capsule production, indicating that *hasC* is not required for hyaluronic acid synthesis and that an alternative source of UDP-glucose is available for capsule production. Nucleotide sequence and deletion mutation analysis of the 5.5 kb of DNA upstream of *hasA* revealed that this region is not required for capsule expression. Many (10 of 23) group A streptococcal strains were found to contain insertion element IS*1239′* approximately 50 nucleotides upstream of the −35 site of the *hasA* promoter. The presence of IS*1239′* upstream of *hasA* did not prevent capsule expression. These results elucidate the molecular architecture of the group A streptococcal chromosomal region upstream of the *has* operon, indicate that *hasABC* are the sole components of the capsule gene cluster, and demonstrate that *hasAB* are sufficient to direct capsule synthesis in group A streptococci.

Group A streptococci (GAS) cause a variety of infections in humans including pharyngitis, invasive infections associated with significant morbidity and mortality, and the unique postinfectious complications of acute rheumatic fever and glomerulonephritis. The GAS hyaluronic acid capsule is a critical virulence factor (20, 22, 33, 37). Three genes, *hasA* (7, 11), *hasB* (12), and *hasC* (4), have been shown to encode enzymes utilized in the synthesis of the polysaccharide. *hasA* encodes hyaluronan synthase, which adds alternating *N*-acetyl-D-glucosamine and D-glucuronic acid residues to form the linear hyaluronic acid polymer (7, 11). *hasB* encodes UDP-glucose dehydrogenase, which forms glucuronic acid from UDP-glucose (12). *hasC* encodes UDP-glucose pyrophosphorylase, which forms UDP-glucose from UTP and glucose-1-phosphate (4). Although the *hasABC* genes are contiguous and form an operon (5), complementation experiments with both GAS and heterologous bacteria have suggested that *hasC* may not be required for capsule synthesis (6).

The small size of the GAS capsule gene region identified to date may reflect the limited genetic requirement for synthesis and export of a linear heteropolymer across the single gram-positive cell membrane. Alternatively, additional genes encoding proteins required for capsule synthesis, regulatory, and export functions may flank the *has* operon, analogous to the genetic organization in several gram-negative bacterial species (3, 14, 26, 34). Genes immediately downstream of *hasC* appear unlikely to be involved in either the synthesis or the expression of capsule (2, 8). The purpose of the present study was to define the genes necessary for GAS hyaluronic acid synthesis by determining the requirement for *hasC* and by characterizing the chromosomal region immediately upstream of the *has* operon.

***hasC* is not required for GAS hyaluronic acid expression.** To derive a GAS *hasC* mutant, initially we amplified by PCR a 630-bp fragment of *hasC* extending from nucleotide 201 to nucleotide 840 with respect to the *hasC* initiation codon with the oligonucleotide primers CCCCCCTCTAGACGAGGAA ATCCTTGTGGTGAC (forward) and CCCCCCAAGCTTCC AACATCGTAACGATTGCC (reverse) and a chromosomal DNA template from the heavily encapsulated M18 GAS strain 87-282. The forward and reverse primers contained the terminal restriction sites *Xba*I and *Hin*dIII, respectively. We cloned the 630-bp amplicon into the temperature-sensitive shuttle vector pJRS233 (30) to form pJHASC. To inactivate the *hasC* gene present in pJHASC, we digested the construct with the restriction endonucleases *Nsi*I and *Sph*I, purified the larger fragment present after digestion, and then ligated a 15-bp 5′-phosphorylated oligonucleotide linker (TCCCCCCCCCGGAT CCGCATG [forward], CGGATCCGGGGGGGGGATGCA [reverse]) to the pJHASC construct via *Nsi*I and *Sph*I compatible ends present in the linker sequence to generate the plasmid pJHASCΔ. Insertion of the linker introduces a *Bam*HI site and multiple stop codons into the *hasC* sequence present in pJHASCΔ.

To demonstrate that the interruption present in the mutant *hasC* allele resulted in loss of UDP-pyrophosphorylase activity, we cloned either the native or mutant *hasC* allele into the expression vector pET-24a (Novagen, Inc. Madison, Wis.) and assayed UDP-glucose pyrophosphorylase activity in the background of *Escherichia coli* DEV6 (kindly provided by the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.), which is deficient in the enzyme (4). Enzyme activity was detected in DEV6 transformed with the expression vector containing the wild-type *hasC* allele, but not in DEV6 either having the vector alone or the vector containing the mutant *hasC* allele, confirming that the mutation in *hasC* resulted in loss of a functional UDP-glucose pyrophosphorylase.

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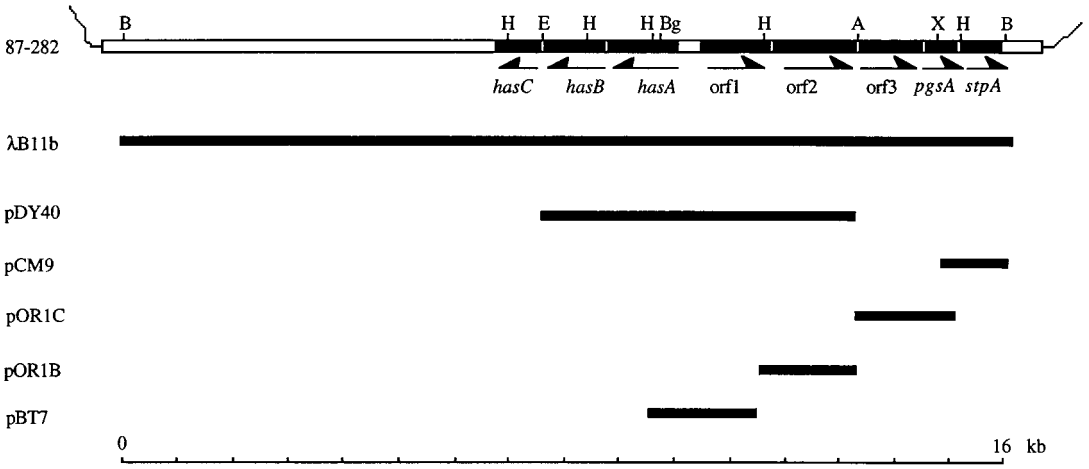

FIG. 1. Schematic map of the GAS capsule gene region and subclones. The *hasABC* genes, the open reading frames upstream of *hasA*, and the subclones from the capsule gene region are shown in black. Arrows indicate the direction of transcription for the *hasABC* genes and the upstream open reading frames. Selected restriction endonuclease sites are indicated: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; E, *Eco*RI; A, *Asp*718; X, *Xba*I.

We replaced the wild-type *hasC* allele in the 87-282 chromosome with the mutant *hasC* allele present in pJHASCΔ by using gene replacement mutagenesis, as previously described (21, 28), to derive strain 282hasCΔ. Southern hybridization demonstrated *hasC* replacement in 282hasCΔ (data not shown). 282hasCΔ had a mucoid colony morphology indistinguishable from that of the parent strain 87-282, suggesting that the two strains produced similar amounts of surface polysaccharide. Measurement of cell-associated hyaluronic acid confirmed that the parent and the *hasC* mutant strain produced similar amounts of polysaccharide (65 ± 1.0 fg/CFU and 118 ± 1.5 fg/CFU, respectively). These results indicate that *hasC* is not required for GAS capsule expression and that sufficient UDP-glucose is present in the cells to permit wild-type levels of hyaluronic acid synthesis in the absence of *hasC* even in a highly encapsulated GAS strain.

Cloning and analysis of 5.5 kb of nucleotide sequence upstream of *hasA* in the GAS strain 87-282 chromosome. Previously, we described the cloning and purification of λB11b, an EMBL3 bacteriophage clone containing the GAS strain 87-282 capsule gene region within a 16-kb insert (2). We subcloned a series of plasmid constructs (Fig. 1) from λB11b and used the plasmids as templates to determine the nucleotide sequence for the 5.5 kb of DNA upstream of *hasA* (Fig. 1). Analysis of the predicted amino acid sequence suggested the presence of five complete open frames, all transcribed divergently from the *has* operon (Fig. 1). The relevant characteristics of these open reading frames, including homologies to sequences in the world database and consensus motifs, are shown in Table 1. None of the first three open reading frames had significant homologies or a consensus motif sufficient to assign a function for the predicted protein. Strong sequence homologies and consensus motifs suggested that orf4 (*pgsA*) encodes a cytidine-diphosphate-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (EC 2.7.8.5) (phosphatidylglycerophosphate synthase) (19, 25, 29) and that orf5 (*stpA*) encodes an ATP binding component of an ATP binding cassette (ABC) transporter (17, 35). Because—with the exception of the most distal gene encoding a component of an ABC transporter—these open reading frames do not have significant homologies to capsule genes in other bacterial species and because ABC transporters are involved in transport of many substrates, it seemed unlikely that the region upstream of *hasA* was involved in GAS capsule expression.

To confirm that the gene products of the open reading frames immediately upstream of *hasA* were not required for polysaccharide capsule synthesis and expression, we derived strain 282orf1-2Δ, in which orf1 and orf2 are deleted. To derive 282orf1-2Δ, we used the oligonucleotide primers CCCTCTA GAAAATCCCGACAATTAAGTC (forward) and CCCGGA TCCCGATTCTCTTAACACTTCACC (reverse), which contain terminal *Xba*I and *Bam*HI restriction endonuclease sites, respectively, to amplify a 2,719-bp amplicon from 87-282 chromosomal DNA template. The PCR product was cloned into vector pJRS233Δ to form plasmid pJORF1-2.*Eco*RV digestion of pJORF1-2, purification of the larger digestion product, and ligation of the purified fragment via the terminal *Eco*RV sites

TABLE 1. Characteristics of open reading frames identified upstream of *hasA*

Open reading frame	Predicted protein		Homology	Consensus motif(s)
	Size (kDa)	No. of amino acids		
orf1	48.2	415	None	None
orf2	49.2	430	None	None
orf3	37.8	342	None	None
pgsA	18.5	166	*pgsA*[a]	DGXXARXXXXXXXXGXXXDXXXD[b] || || | | | DGYLARKWHVVSNFGKFADPLAD
stpA	33.2	297	ABC[c]	GXXXXGKS[d] | ||| GHNGSGKS
				LSGGXXXRVXIA[e] |||| || || LSGGQKQRVAIA

[a] Gene encoding phosphatidylglycerophosphate synthase (EC 2.7.8.5). Greatest homology is to *Bacillus subtilis pgsA* (25).

[b] Consensus motif for phosphatidyltransferases (19, 29). Lower line corresponds to amino acids 52 to 74 of GAS *pgsA*.

[c] ABC transporters. Greatest homology is to the ATP binding component of a putative peptide transporter in *Mycoplasma pneumoniae* (18).

[d] Consensus motif for P-loop of nucleotide binding proteins (35). Lower line corresponds to amino acids 57 to 64 of GAS *stpA*.

[e] Signature motif for ATP binding proteins of ABC transporters (17). Lower line corresponds to amino acids 158 to 169 of GAS *stpA*.

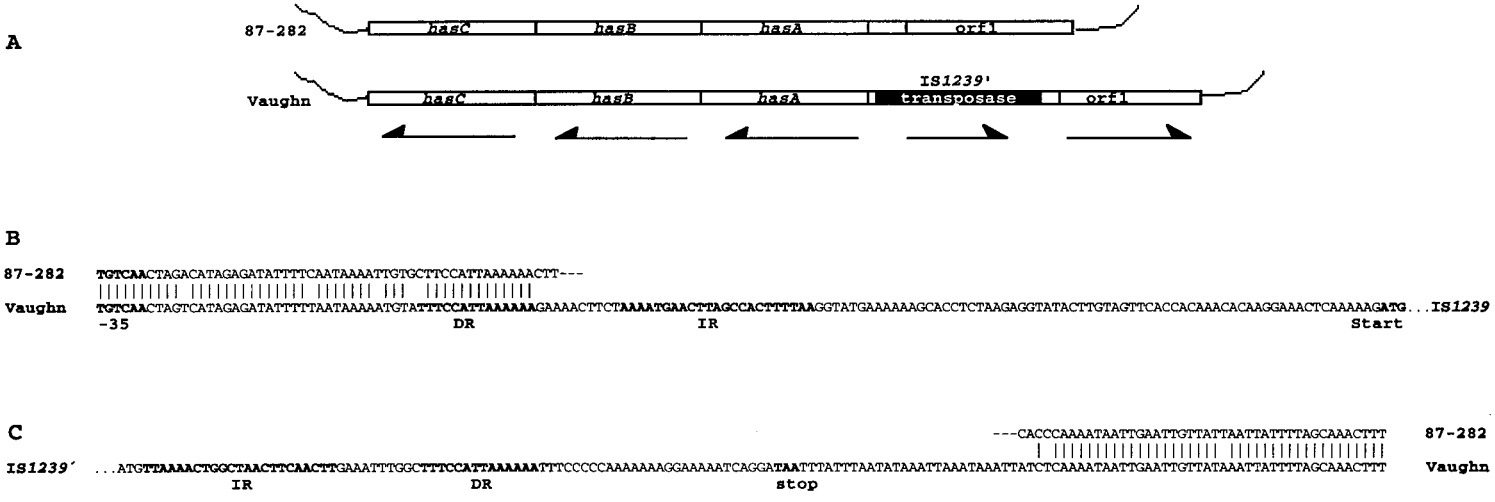

FIG. 2. Schematic representation comparing the 87-282 and Vaughn capsule gene regions and sequence analysis defining the boundaries and insertion site of the insertion element IS*1239′*. (A) Comparison of the site of IS*1239′* insertion in GAS strain Vaughn to the homologous chromosomal region in GAS strain 87-282. The insertion element is shown in black. The genes *hasABC*, the putative insertion element transposase, and orf1 are identified; arrows indicate the direction of gene transcription. (B) Comparison of the nucleotide sequences beginning 71 nucleotides upstream of the *hasA* initiation codon in GAS strains 87-282 and Vaughn. Alignment of the homologous sequences is shown; identical nucleotides are indicated by vertical bars. The *hasA* −35 promoter site is shown in boldface type and indicated. Inverted (IR) and direct (DR) repeat sequences are shown in boldface type. The putative initiation codon for the IS*1239′* transposase is indicated (Start). Dashes in the 87-282 sequence indicate continuity with the 87-282 sequence in panel C. Dots in the Vaughn sequence indicate the continuation of the IS*1239′* nucleotide sequence, which is not shown. (C) Comparison of the nucleotide sequences approximately 250 bp upstream of the putative initiation codon for orf1 in GAS strains 87-282 and Vaughn. Alignment of the homologous sequences is shown; identical nucleotides are indicated by vertical bars. Dashes in the 87-282 sequence indicate continuity with the 87-282 sequence shown in panel B. Dots in the Vaughn sequence indicate preceding nucleotides present in IS*1239′*. Inverted (IR) and direct (DR) repeat sequences are shown in boldface type. The putative termination codon for the IS*1239′* transposase is indicated (stop).

generated plasmid pJORF1-2Δ. The insert in pJORF1-2Δ was comprised of a 355-bp fragment that included 95 bp of orf1 upstream sequence plus the first 260 nucleotides of orf1 linked to a 342-bp fragment that contained 250 bp of the orf2 3′ terminus plus an additional 92 nucleotides of downstream sequence. In linking these regions in the pJORF1-2Δ construct, 989 bp of orf1 and 1,037 bp of orf2 were deleted. The mutant orf1-orf2 allele present in pJORF1-2Δ was introduced into the 87-282 chromosome by allelic exchange mutagenesis (21, 28) to derive strain 282orf1-2Δ. Southern hybridization analysis confirmed gene replacement in 282orf1-2Δ. Deletion of orf1 and orf2 had no apparent effect on capsule production; both the parent and the mutant strain had a mucoid colony morphology on blood agar medium and similar amounts of cell-associated hyaluronic acid (65 ± 1.0 fg/CFU and 74 ± 2.8 fg/CFU, respectively) (33). These results indicate that the two open reading frames immediately upstream of *hasA* are not required for GAS capsule expression.

To investigate whether the region upstream of *hasA* in the mucoid strain 87-282 is conserved in other GAS strains, we used pDY40 to probe a Southern blot of *Hin*dIII-digested genomic DNA from 23 GAS strains including a variety of clinical isolates and M protein types. The Southern blot demonstrated that 10 of 23 GAS strains contained an additional 1.1 kb of DNA immediately upstream of *hasA* (data not shown). We amplified this region from the type 24 GAS strain Vaughn by using PCR and the primers AACGGATAGGTCTGTGCT AAC (forward) and TTATTCAACAACATCGACCTG (reverse). Compared to the 87-282 sequence from this region, the sequence obtained from the strain Vaughn PCR product contained approximately 1.1 kb of additional DNA, of which 969 nucleotides were 99% identical to the sequence of the GAS insertion element IS*1239* (23). The only significant difference was an additional 36 nucleotides present in the strain Vaughn element which extended the carboxy terminus of the putative transposase by 12 additional amino acids. Because the insertion element present in strain Vaughn appears to be a slightly larger variant of IS*1239*, we have designated it IS*1239′*. Further comparison of the nucleotide sequence between GAS strains 87-282 and Vaughn localized IS*1239′* integration to a locus 46 nucleotides upstream of the −35 site of the *hasA* promoter (Fig. 2). Measurement of cell-associated hyaluronic acid in a sample of 23 GAS strains demonstrated no correlation between the presence of the insertion sequence upstream of *hasA* and the amount of cell-associated polysaccharide. This observation is consistent with studies showing that full activity of the *has* operon promoter requires no more than 12 nucleotides of flanking sequence upstream of the −35 site (1) and supports the sequence analysis suggesting that genes upstream of *hasA* are unlikely to be involved in capsule expression.

The results of these studies provide evidence that the GAS capsule gene region is comprised solely of the *hasABC* genes and that only *hasAB* are uniquely required for capsule production. Additional proteins must be involved in the biosynthesis of hyaluronic acid—the enzymes involved in the synthesis of UDP–*N*-acetylglucosamine, for example—but these functions are likely to be shared with other synthetic or metabolic pathways in the cell. The *hasC* gene product, UDP-glucose pyrophosphorylase, is not required for hyaluronic acid synthesis, indicating that an alternative source of UDP-glucose is available for capsule production. Epimerization of UDP-galactose to UDP-glucose has been reported for *Streptococcus pneumoniae* (10), but such a pathway seems unlikely in GAS that do not contain galactose as a cell surface component. GAS lipoteichoic acid has been reported to be glucosylated (16). Since glucosylation of lipoteichoic acid requires UDP-glucose in other bacterial systems (36), it is possible that a *hasC* homolog in GAS is clustered with genes involved in lipoteichoic acid synthesis.

The capsule gene cluster in GAS is quite similar to that of *S. pneumoniae* type 3, in which the genes uniquely required for capsule production comprise a four-gene cluster that includes three genes analogous to *hasABC* (9). Both the GAS and *S. pneumoniae* type 3 capsule gene regions are unusually small compared to most other encapsulated bacteria that contain multiple genes involved in capsule synthesis and surface expression (3, 13, 15, 26, 27 31, 34). The limited genetic requirement for capsule expression in GAS and *S. pneumoniae* type 3 likely reflects the relative simplicity of the capsular polysaccharide that these organisms produce and supports the prediction that this family of glycosyl transferases exports polysaccharide in the process of polymerization (24, 32).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been submitted to GenBank under accession no. AF082738 and AF082865.

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