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# Analysis of a Second Bacteriophage Hyaluronidase Gene from *Streptococcus pyogenes*: Evidence for a Third Hyaluronidase Involved in Extracellular Enzymatic Activity

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**The hyaluronidase gene (*hylP2*) from a second group A streptococcal bacteriophage was isolated from ATCC T-type-22 hyaluronidase-producing strain 10403, a strain known to produce increased amounts of extracellular hyaluronidase. Sequence analysis of *hylP2* and alignment with the previously described bacteriophage hyaluronidase gene (*hylP*) showed a high degree of similarity; however, *hylP2* had deletions of regions specifying 34 amino acids. Twenty-eight of the deleted amino acids were in a region of HylP containing a series of collagen-like Gly-X-Y repeating units. By employing primers for both *hylP* and *hylP2*, PCR amplification resulted in fragments of appropriate sizes in 97% of the strains tested, with some strains producing two fragments, indicating the presence of at least two phages. When the *hylP2* gene was introduced via a plasmid vector into a non-hyaluronidase-producing *Streptococcus pyogenes* strain, this strain was still unable to produce extracellular hyaluronidase, although intracellular hyaluronidase was present. These results, along with the absence of a typical N-terminal signal peptide, indicate that HylP2 is unable to be secreted into the extracellular milieu. Examination of more than 100 strains for production of hyaluronidase showed that only 23% of the strains produced extracellular hyaluronidase. One of these strains (strain 10403) contains a single bacteriophage hyaluronidase gene (*hylP2*) which, when inactivated by allelic replacement, still produces large amounts of extracellular hyaluronidase. These results suggest the presence of a different hyaluronidase gene encoding a protein that is actively secreted into the extracellular milieu.**

Hyaluronidase production by *Streptococcus pyogenes* and other pathogenic streptococci (groups B, C, and G and *Streptococcus pneumoniae*) has been a subject of interest since early reports of McClean (25), who described an extracellular factor present in the culture supernatants of these organisms capable of cleaving hyaluronic acid. The substrate of hyaluronidase is hyaluronic acid, a sugar polymer composed of alternating *N*-acetylglucosamine and glucuronic acid residues. Hyaluronic acid is found in the ground substance of human connective tissue and the vitreous of the eye and also is the sole component of the capsule of group A streptococci. The capsule has been shown to be an important virulence factor of this organism by virtue of its ability to resist phagocytosis (35, 36). Production by *S. pyogenes* of both a hyaluronic acid capsule and hyaluronidase enzymatic activity capable of destroying the capsule is an interesting, yet-unexplained, phenomenon.

Production of hyaluronidase by group A streptococci has been suggested to aid the organism in its spread through the connective tissue (25); hence, hyaluronidase has been designated as one of the spreading factors of microbial origin. Although generally considered an extracellular product, it is known that intracellular forms of hyaluronidase which are encoded by phages integrated in the host chromosome may exist. Relatively little is known about the extracellular hyaluronidase, and it was originally thought to be associated with only two serotypes (9). Subsequent reports suggested that hyaluronidase is not serotype related but a property of the particular strain (2). Much of the present information available in the literature concerning the hyaluronidases of group A streptococci relates to the phage-specific hyaluronidase (1–4, 22, 26). The sequence of a hyaluronidase gene (*hylP*) from the group A streptococcal temperate bacteriophage H4489A was reported previously from this laboratory (19). Of particular interest in the deduced amino acid sequence of this gene was the lack of a signal peptide in the protein, suggesting that when bacteriophage hyaluronidase is detected extracellularly, it is released following liberation of phage particles from lysogenic cells. The sequence of HylP has no homology to the sequences of the extracellular hyaluronidases recently sequenced from *S. pneumoniae* (7), a group B streptococcus (24), and *Clostridium perfringens* (8). In this report, we describe the nucleotide and deduced amino acid sequences of a second bacteriophage hyaluronidase gene which was isolated from the ATCC T-type-22 group A streptococcus strain 10403, a strain known to produce high levels of hyaluronidase. A comparison of the properties of the two bacteriophage hyaluronidases is presented along with evidence indicating the existence of an extracellular hyaluronidase produced by strain 10403.

## MATERIALS AND METHODS

**Enzymes and chemicals.** Restriction enzymes, DNA-modifying enzymes, M13 vector DNA, and the M13 17-base primer were purchased from either Bethesda Research Laboratories (Gaithersburg, Md.) or Promega (Madison, Wis.) and used as described in the manufacturer's instructions. Dephosphorylated *Bam*HI-cut pUC13 vector was obtained from the Molecular Biology Division, Pharmacia, Inc. (Piscataway, N.J.). The [$\alpha$-$^{32}$P]dATP and $\alpha$-$^{35}$S-dATP were from New England Nuclear Corp. (Boston, Mass.).

**Hyaluronidase assay.** The assay of hyaluronidase activity was done by the use of brain heart infusion-bovine albumin fraction V-hyaluronic acid agar (BHB) plates prepared as described previously (20). Poor growth of some strains on BHB could be overcome by substituting Todd-Hewitt agar as the basal medium. The assay of liquid preparations such as culture supernatants was also carried out with BHB agar, modified by the addition of 0.1% sodium azide to prevent bacterial growth (20).

**Cloning of the hyaluronidase gene from group A streptococcus strain 10403.** Total cellular DNA from the group A streptococcus ATCC strain 10403 (T type

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22) was isolated as described by Huang et al. (18). Partial digestion of the DNA with *Sau*3A, separation into 2.0-kb fractions by gel electrophoresis, and extraction from agarose were done by established procedures. Sized fractions (1 to 6 kb) were ligated with *Bam*HI-dephosphorylated pUC13 and transformed into *Escherichia coli* DH5α-competent cells (Bethesda Research Laboratories). Recombinant colonies were transferred onto fresh plates and stabbed into BHB media for the assay of hyaluronidase activity.

**Southern hybridization.** DNA transfer, nick translation, and hybridization were performed by the method of Southern (31) as described by Sambrook et al. (27). Nonradioactive detection of DNA following transfer to Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.) was by the Genius System as described by the manufacturer (Boehringer Mannheim Corp., Indianapolis, Ind.).

**Nucleotide sequencing.** A 3.0-kb *Xba*I DNA fragment to be sequenced was subcloned into M13 vectors prior to sequencing. The nucleotide sequence was determined by the dideoxy chain termination procedure (28) with Sequenase as described by the manufacturer (Pharmacia). Directionally deleted DNA clones were prepared by *Exo*III digestion as described by Henikoff (16) or by degradation with nuclease *Bal*31 by a modification of the technique of Gilmore et al. (12). Additional clones were prepared, as required, by forced cloning of fragments. The DNA sequence was confirmed on both strands.

**Computer analysis.** Initial computer analysis of sequence information was by the James M. Pustell DNA-protein sequencing program from International Biotechnologies Inc. (New Haven, Conn.). Additional analysis was carried out with the Genetics Computer Group (University of Wisconsin) sequence analysis software (10).

**PCR analysis.** More than 100 group A streptococcal strains from the collection of J. J. Ferretti (University of Oklahoma Health Sciences Center, Oklahoma City) of different serotypes, having different disease associations and widespread geographical locations (Canada, England, Germany, New Zealand, Japan, United States, Thailand, and France), were analyzed for the presence of hyaluronidase genes by amplification with the PCR with crude cell lysates as described previously (21). Oligonucleotide primers were prepared by the Molecular Biology Resource Facility of the Oklahoma Center for Molecular Medicine, Oklahoma City. Primers utilized for amplification of the hyaluronidase genes were as follows: primer 1, ATGACTGAAAATATACCATTAAGA; primer 2, ATGA GTGAAAATATACCGCTGCGA; primer 3, CTATTTTTTTAGTATGAGTT TTTT; primer 4, CTTGAGGGTGAGATAGGTTTTGAG; primer 5, ATTAA GTGCCGAGGAAAAATTAGG. Primers 1 and 2 corresponded to the 5′ ends of *hylP* and *hylP2*, respectively. Primer 3 was the 3′ end of *hylP* and *hylP2*, which showed 100% identity in the last 24 bp. Primers 4 and 5 were internal to the hyaluronidase structural genes, homologous to the sequence from *hylP*, and shared greater than 80% identity to the sequence in *hylP2*.

PCR was carried out in 25-μl volumes containing 200 μM each deoxynucleotide (dATP, dTTP, dGTP, and dCTP), 0.5 μM each primer, 1 U of Perfect-Match (Stratagene, La Jolla, Calif.), and 1 U of *Taq* DNA polymerase (Promega). Buffer was added as described in the manufacturer's instructions. Reaction mixes were covered with 25 μl of mineral oil and placed in an automated thermal cycler (M. J. Research, Inc., Watertown, Mass.). Amplification conditions varied depending on the particular primers. For primers 1 (or 2) and 3, conditions were 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and extension at 72°C for 1 min for 30 cycles. When using the internal primers (primers 4 and 5), amplification conditions were 1 min of denaturation at 94°C, 1 min of annealing at 62°C, and extension at 72°C for 1 min for 30 cycles. In both instances, a further extension for 5 min at 72°C was carried out after completion of the 30 cycles. After amplification, 5 μl of the reaction mix was assayed on an agarose gel in the presence of 0.5 μg of ethidium bromide per ml (27).

**Inactivation of *hylP2* in strain 10403.** To ascertain the role of *hylP2* in the production of extracellular hyaluronidase activity, an isogenic mutant was constructed in which the *hylP2* gene was inactivated. Initial manipulations were carried out in *E. coli* with the cloned *hylP2*-containing fragment. A 1.5-kb *Tha*I-*Pst*I fragment was subcloned, from the 3.0-kb *Xba*I fragment, into pUC18. An 802-bp *Cla*I-*Bcl*I fragment internal to *hylP2* was removed, and the ends were blunted with mung bean nuclease and Klenow fragment. An erythromycin resistance marker obtained from the *Lactococcus lactis* vector pIL252 (29) was inserted in place of the deleted region. The construct, *hylP*::*erm*$^+$, was amplified by PCR. Following amplification, the DNA fragment had 262 and 324 bp at the ends homologous to the strain 10403 (bacteriophage) chromosomal region. Amplified DNA was purified and electrotransformed (30) into strain 10403. Two erythromycin-resistant clones were utilized in further analysis. The mutants were assayed for production of hyaluronidase activity on BHB media, with replacement of *hylP2* by the erythromycin gene confirmed by PCR (21) and Southern blot analysis (27).

**Cloning of the *hylP2* gene into group A streptococcus strain K56.** The *hylP2* gene was cloned into the *E. coli*-streptococcus shuttle vector pAT28 (33). The putative promoter region was also cloned with the structural gene. The vector containing the cloned *hylP2* gene was then introduced into group A streptococcus strain K56 by electrotransformation (30). Transformants were selected by spectinomycin resistance; spectinomycin selection was utilized in subculturing to ensure maintenance of the plasmid. Spectinomycin-resistant clones were screened for the production of extracellular and intracellular hyaluronidase activity. Cell lysates for the assay of intracellular activity were prepared as follows: overnight Todd-Hewitt broth cultures were washed twice with fresh Todd-Hewitt broth, resuspended in distilled water, and frozen and thawed twice before being broken by a French press.

**Nucleotide sequence accession number.** The sequence data published here have been submitted to GenBank under accession number U28144.

## RESULTS

**Cloning the hyaluronidase gene from group A streptococcal strain 10403.** *Sau*3A-digested DNA was ligated into pUC13 before transformation into competent *E. coli* DH5α cells. After growth of transformants, 1,352 colonies were screened for the production of hyaluronidase on BHB agar plates. Four transformants which produced hyaluronidase activity were obtained. Restriction enzyme analysis indicated that three of the clones contained identical 4.5-kb fragments. The other cloned fragment was 3.0 kb in size and appeared to be contained within the 4.5-kb fragment obtained from the other clones. A 3.0-kb *Xba*I fragment was subcloned from the 4.5-kb fragment into the plasmid vectors pUC18 and pUC19 as well as the bacteriophage M13 vectors mp18 and mp19. All transformants obtained produced hyaluronidase activity, indicating that the cloned region contained not only the hyaluronidase gene but also a region able to function as a promoter in *E. coli*.

The 3.0-kb *Xba*I fragment was purified and used as a probe in Southern hybridizations with DNA isolated from *S. pyogenes* 10403. The purified probe hybridized to total cellular DNA from strain 10403, showing that the cloned DNA originated from this strain (results not shown).

**Nucleotide sequence of *hylP2*.** The sequenced *Xba*I fragment was 2,974 bp in length. Examination of the sequence revealed three open reading frames (ORFs), two of which were incomplete. ORF1 lacked the N terminus of the peptide, while ORF2 lacked the C-terminal portion. The two partial ORFs possessed similarity to the DNA regions surrounding the *hylP* gene (19). The complete ORF encoded the hyaluronidase gene *hylP2*, which when cloned independently was sufficient to encode hyaluronidase activity. The sequence of *hylP2* (Fig. 1) is 1,014 bp in length, encoding a 337-amino-acid protein with a deduced molecular weight of 36,432.

Computer analysis of *hylP2* showed 66.5% identity and 77.5% similarity to the previously reported *hylP* gene from bacteriophage H4489A (19). These two hyaluronidase genes differ in length by 102 bp (34 amino acids). Alignment of the sequences to show maximal similarity and least number of gaps (10) suggests that the size difference occurs by deletion of 28, 4, 1, and 1 amino acids throughout the protein (Fig. 2). No similarity was found between *hylP2* and the hyaluronidase genes from *Streptococcus agalactiae* (24), *S. pneumoniae* (7), or *C. perfringens* (8).

**PCR amplification of streptococcal hyaluronidase genes.** By use of crude cell lysates, more than 100 different streptococcal strains were screened for the presence of bacteriophage hyaluronidase genes (21). Southern blot analysis, using probes derived from *hylP* and *hylP2*, confirmed that the DNA obtained following PCR amplification was derived from a hyaluronidase gene.

A DNA fragment was amplified from plasmid pSF49 containing the cloned *hylP* gene (19) and also from bacteriophage H4489A DNA with primers 1 and 3, which corresponded to the 5′ and 3′ ends of *hylP*, respectively. Conversely, when primers 2 and 3 were used, corresponding to the 5′ and 3′ ends of *hylP2*, amplification of DNA fragments occurred with DNA from strain 10403 and plasmids containing the cloned *hylP2* gene. As expected from the sequence of the cloned genes, there was a variation in size between the DNAs amplified from the two parental strains. This difference in the sizes of the two fragments verified that the deletion observed within the *hylP2*

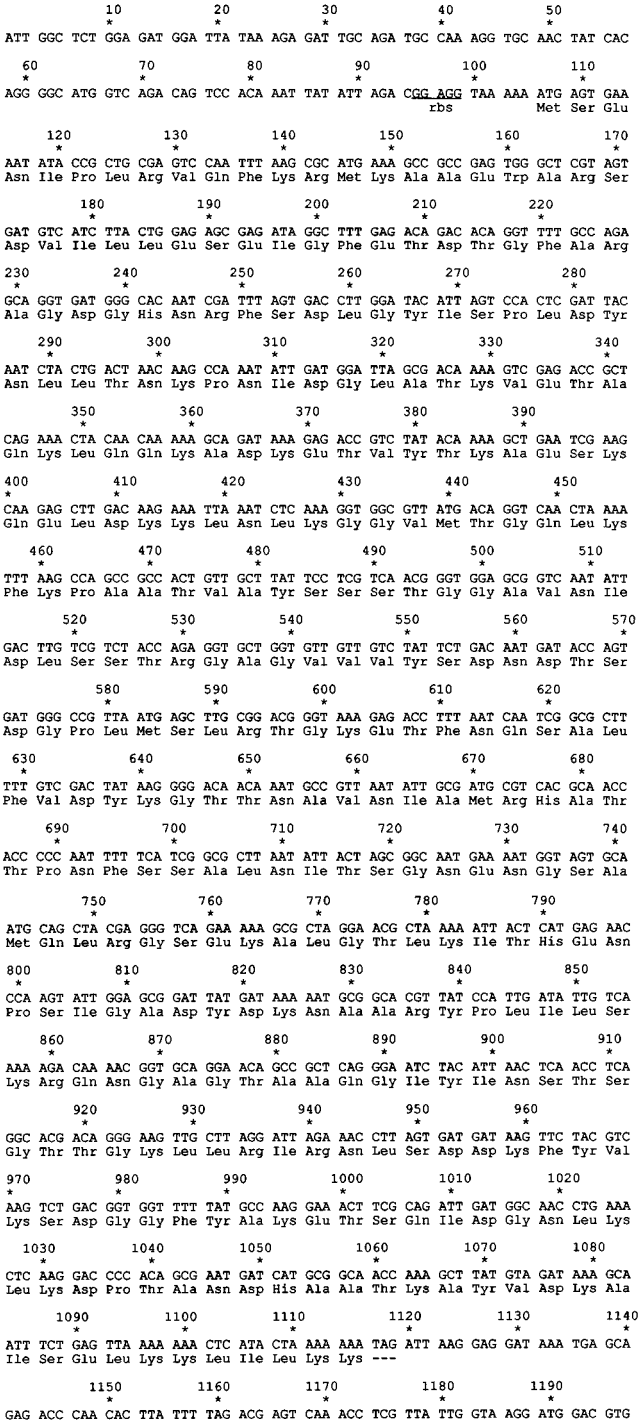


FIG. 1. Nucleotide sequence of the hyaluronidase gene (*hylP2*) from group A streptococcus strain 10403. The deduced amino acid sequence of HylP2 is shown below the nucleotide sequence. A putative ribosome binding site (rbs) is underlined.

gene had not occurred during cloning or subsequent manipulations. By use of primers internal to the hyaluronidase genes, DNA fragments from both *hylP* and *hylP2* were able to be amplified. The primers (i.e., primers 4 and 5) were positioned such that amplified DNA would cover 99 of the 102 bp deleted (Fig. 2B). By use of the PCR with primers 4 and 5, we were able to screen group A streptococcal strains for the hyaluronidase genes as well as to differentiate between the two hyaluronidase gene types on the basis of the variation in size of the amplified DNA fragments (Fig. 3).

Strains of group A streptococci of different M types and from a wide variety of sources were examined for the presence of hyaluronidase genes by use of primers 4 and 5. The size of the fragment amplified allowed for determination of the type of hyaluronidase gene (*hylP* or *hylP2*) present in the representative strains. Where possible, at least three isolates from each serotype were analyzed by PCR. Of the strains tested, 97% (115 of 119 strains) amplified a DNA fragment. Approximately 30% of the strains amplified a fragment of the size expected for *hylP*, while 54% amplified a fragment equal in size to that of *hylP2*. Within some M types, all three of the strains tested amplified a fragment corresponding to *hylP* (M types 1, 6, and 60) or *hylP2* (M types 5, 17, 18, 30, 36, 53, and PT2841). Other M types showed variations between strains which have either *hylP* or *hylP2*. Some strains (14%) showed amplification of two fragments corresponding to the sizes of both *hylP* and *hylP2*, suggesting the presence of both hyaluronidase genes within the same strain (Fig. 3). Only four strains (0.3%) did not amplify a DNA fragment by use of the internal hyaluronidase primers. Although a large proportion of the streptococcal strains examined contained a bacteriophage hyaluronidase gene, only 23% of the strains tested produced any detectable hyaluronidase activity by our assay.

**Inactivation of *hylP2* in strain 10403 and expression of *hylP2* in strain K56.** As was found with *hylP* (19), the sequence of *hylP2* did not encode an N-terminal region characteristic of a signal peptide. It was therefore of interest to determine whether *hylP2* plays a role in the production of extracellular hyaluronidase activity. An isogenic mutant of strain 10403 was constructed by replacement of an internal portion of *hylP2* with an erythromycin resistance gene. PCR and Southern blot analysis confirmed that the *hylP2* gene had been interrupted in one of the mutants. The mutant strain did not show a decrease in extracellular hyaluronidase activity as detected by our assay, suggesting that a separate gene encodes this enzymatic activity.

To ascertain whether *hylP2* was able to be secreted from a streptococcal cell, the *hylP2* gene was cloned into the *E. coli*-streptococcus shuttle vector pAT28 and introduced into *S. pyogenes* K56 by electrotransformation. Strain K56 was chosen for this study since it did not produce hyaluronidase activity and did not amplify a DNA fragment by use of primers for *hylP* or *hylP2*, suggesting that K56 lacks at least these two hyaluronidase genes and any other functional hyaluronidase gene. When *hylP2* was introduced into strain K56, no extracellular hyaluronidase activity was detected. However, when the cells were broken with the French press, hyaluronidase activity could be detected in the intracellular milieu, indicating that the *hylP2* gene was being expressed in this host strain.

## DISCUSSION

Streptococcal hyaluronidase has long been designated as a spreading factor because of its ability to attack the hyaluronic acid present in the cement substance of host tissues. This hyaluronidase has always been presumed to be an extracellular protein, and it was our original intent to clone and sequence this gene; however, two attempts with separate group A strains resulted in the cloning of two different bacteriophage hyaluronidase genes, *hylP* and *hylP2*. Examination of more than 100 group A strains from different serotypes for their ability to produce extracellular hyaluronidase showed that only 23% of the strains produced extracellular hyaluronidase. However,

A)

*hyl*P MTENIPLRVQFKRMSADEWARSDVILLEGEIGFETDTGFAKFGDGQNTFSKLKYLTGPKGPKGDT
*hyl*P2 MsENIPLRVQFKRMkAaEWARSDVILLESEIGFETDTGFARaGDGHNrFSdLgYI**********

*hyl*P GLQGKTGGTGPRGPAGKPGTTDYDQLQNKPDLGAFAQKEETNSKITKLESSKADKSAVYSKAESK
*hyl*P2 ******************SplDYNlLtNKPNIDGLAtKvETaqKLqq****KADKetVYtKAESK

*hyl*P IELDKKLSLTGGIVTGQLQFKPNKSGIKPSSSVGGAINIDMSKSEGAAMVMYTNKDTTDGPLMIL
*hyl*P2 qELDKKLnLkGGVMTGQLkFKPa*atVaySSStGGAVNIDlSstrGAGVVVYsDnDTsDGPLMsL

*hyl*P RSDKDTFDQSAQFVDYSGKTNAVNIVMRQPSAPNFSSALNITSANEGGSAMQIRGVEKALGTLK
*hyl*P2 RtGKETFNQSAlFVDYkGtTNAVNIaMRHAttPNFSSALNITSGNEnGSAMQLRGsEKALGTLK

*hyl*P ITHENPNVEAKYDENAAALSIDIVKKQKGGKGTAAQGIYINSTSGTAGKMLRIRNKNEDKFYVGP
*hyl*P2 ITHENPsIGAdYDkNAArypLiLsKRQnGA*GTAAQGIYINSTSGTtGKLLRIRNlsDDKFYVks

*hyl*P DGGFHSGANSTVAGNLTVKDPTSGKHAATKDYVDEKIAELKKLILKK-
*hyl*P2 DGGFyaketSqIdGNLkLKDPTandHAATKaYVDkaIsELKKLILKK-

B)

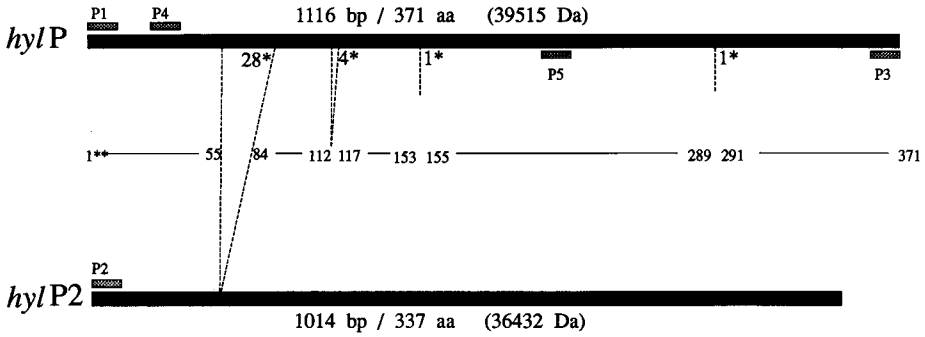


FIG. 2. (A) Comparison of the amino acid sequences of HylP and HylP2. Identical amino acids are shown in bold type. Conservative changes are indicated by uppercase letters; nonconserved amino acids are in lowercase letters in HylP2. The 10 repeats of the collagen triplet (Gly-X-Y) in HylP are underlined. Proposed positions of the deleted amino acids in HylP2 are indicated by asterisks. These gaps constitute deletions consisting of 28, 4, 1, and 1 amino acids. (B) Schematic alignment of *hylP* and *hylP2* indicating the regions where possible deletions have occurred. A single asterisk indicates the number of amino acids deleted at the particular region. A double asterisk indicates the amino acid position relative to the amino acid sequence of HylP. The positions of the primers (P1 to P5) used in the PCR studies are indicated. aa, amino acids.

PCR analysis with primers which amplified both *hylP* and *hylP2* genes indicated that 97% of the strains contained one or the other, or sometimes both, of these genes. These results indicate the presence of multiple genes which encode hyaluronidase activity, an observation in agreement with previous studies which suggested that hyaluronidases are isoenzymes (11, 13, 14, 17). In these previous studies, hyaluronidases were obtained from culture filtrates of strains, and it is not known whether spontaneous lysis of cells which contributed phage hyaluronidase to any other hyaluronidase present could have occurred. Our findings are also consistent with the demonstration of different serological types of phage hyaluronidases (5, 6, 23). Finally, these results confirm earlier observations of multiple lysogeny and a high percentage of lysogenic bacteriophages present in group A streptococci (34).

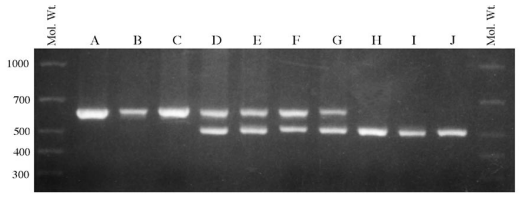


FIG. 3. PCR screening of group A streptococcal strains for hyaluronidase genes by use of primers 4 and 5 showing amplification of *hylP*, *hylP2*, or *hylP* and *hylP2*. Lanes: A, strain GT8760 (M type 49); B, strain D733 (M type 39); C, strain F711 (M type 3); D, strain NZ131 (M type 49); E, strain D904 (M type 3); F, strain D336 (M type 61); G, strain 3257 (M type 78); H, strain F690 (M type 9); I, strain F418 (M type 60); J, strain 10403 (T type 22). Molecular weight marker sizes are indicated on the left of the figure.

In a previous study, we reported the cloning and sequencing of a hyaluronidase gene (*hylP*) from the streptococcal bacteriophage H4489A (19). The *hylP* gene encoded a protein of 39,515 Da which lacked an N-terminal signal peptide essential for secretion as an extracellular protein. Partial ORFs were found upstream and downstream of *hylP*; however, the function of the proteins encoded by these ORFs remains unknown. In the current study, we cloned and sequenced a second hyaluronidase gene, *hylP2*, isolated from *S. pyogenes* 10403 (T type 22), known to produce high levels of extracellular hyaluronidase. The *hylP2* gene encoded a protein of 36,432 Da which was characterized by the lack of a sequence resembling an N-terminal signal peptide. This information, along with the fact that the ORFs upstream and downstream of *hylP2* were

similar to those flanking the bacteriophage *hylP* gene, suggested that *hylP2* was also derived from a bacteriophage. Attempts at obtaining a bacteriophage from strain 10403 following mitomycin induction have to date been unsuccessful, leading us to speculate that this unknown phage may be defective. Alternatively, an appropriate host may not have been utilized since previous studies have indicated that the frequency of detecting lysogeny is increased by utilizing different streptococcal host strains (34).

It is noteworthy that the hyaluronidase (HylP) from phage H4489A contains a series of 10 Gly-X-Y amino acid triplets, closely resembling the characteristic repeating sequences found in collagen (32). Stern and Stern (32) speculated that residues within these repeats have the potential, in eukaryotes, to undergo posttranslational hydroxylation and may be involved in the stabilization of trimeric structure. However, this region is missing from HylP2, suggesting that these repeats are not important for the enzymatic activity of hyaluronidase. The origin of this 90-bp region of DNA in the hyaluronidase genes is unknown; however, it is clearly widespread, since the sequence is present in a number of strains as determined by PCR analysis.

A possible role in human disease of this collagen-like repeat found in the bacteriophage hyaluronidase could be the induction of antibodies which may cross-react with tissue collagen and result in the polyarthritis often associated with rheumatic fever. We examined 40 group A isolates from patients with rheumatic fever and found that approximately 30% of these strains possessed the *hylP* gene encoding the collagen-like repeat region. Because of the lack of both complete case history information and serum samples of the patients from which these strains were obtained, it was not possible to make any association between the presence of antibody to HylP and polyarthritis symptoms.

The contribution of bacteriophage-encoded hyaluronidases to the extracellular hyaluronidase activity produced by these organisms is difficult to quantitate since so little is known about the extracellular hyaluronidase enzyme. We have been unable to detect intracellular bacteriophage hyaluronidase in many of the strains that contain either bacteriophage *hylP* or *hylP2* genes, presumably because these genes are not expressed in the prophage state. When a cloned *hylP2* gene was introduced into group A strain K56, which produced no extracellular hyaluronidase and lacked both bacteriophage *hylP* and *hylP2* genes, no extracellular hyaluronidase was found. However, following cell breakage, intracellular hyaluronidase was readily detected. These observations provide evidence that even when *hylP2* is fully expressed, the bacteriophage enzyme is unable to be secreted by these organisms. Recently, the sequence of hyaluronidase from *S. pneumoniae* has been reported to contain no signal sequence, with extracellular release occurring upon autolysis of the organism in a manner similar to that of the release of pneumolysin (7). Although a similar autolytic mechanism has not been reported in the group A streptococci, individuals infected with group A streptococci containing a lysogenic phage are able to mount an antibody response directed against the bacteriophage hyaluronidase (15). Although the phage hyaluronidase may be tightly bound to, or constitute, an integral element of the phage, the major portion of the hyaluronidase produced by infected strains appears to be free and unassociated with bacteriophage particles (4). This suggests that during an infection, lysogenic bacteriophages may be induced, resulting in lysis of the group A streptococcus and liberation of phage particles and free hyaluronidase. It is conceivable that the unassociated bacteriophage hyaluronidase, liberated upon lysis of the host, may aid in dispersion of the infecting organism from its initial site of infection, in essence acting as a virulence factor for the host bacterium. The role of the hyaluronidase for the bacteriophage, however, is most likely more direct. Group A streptococci have a hyaluronic acid capsule that may mask receptors for a phage. Bacteriophages with an associated hyaluronidase, either bound or as an integral component, would be able to penetrate the capsule. Once the capsule has been penetrated, the phage would gain access to the appropriate receptors and be able to infect the new streptococcal cell.

Inactivation of the *hylP2* gene in strain 10403 has no effect on the high levels of extracellular hyaluronidase known to be produced by this strain. Thus, strain 10403 possesses an additional hyaluronidase gene which we believe to be chromosomal in origin and responsible for the secreted hyaluronidase. Whether this gene is present in all strains of group A streptococci is not known since the majority of strains do not produce detectable extracellular hyaluronidase activity. Currently, several conflicting data exist in the literature concerning which serotypes of group A streptococci actually produce hyaluronidase, whether production is limited to only a few serotypes (9), or whether all serotypes have strains with the ability to elaborate hyaluronidase (1, 2). The eventual cloning and sequencing of a chromosomal hyaluronidase gene from group A streptococci should provide important information about its epidemiology and role as a spreading factor in streptococcal diseases.

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