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Cloning and Nucleotide Sequence of the *Streptococcus pneumoniae* Hyaluronidase Gene and Purification of the Enzyme from Recombinant *Escherichia coli*

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A gene bank of *Sau*3A1-generated *Streptococcus pneumoniae* type 23 DNA fragments was constructed in *Escherichia coli* K-12 with the low-copy-number cosmid vector pOU61cos. Clone lysates were screened by immunoblotting using a mouse antiserum raised against a crude pneumococcal hyaluronidase preparation. One immunoreactive clone was isolated, and it produced high levels of hyaluronidase activity. This clone contained a recombinant cosmid (designated pJCP800) with an approximately 35-kb DNA insert, and the putative hyaluronidase coding sequence was subcloned into pBluescript SK as a 3.8-kb *Pst*I-*Cla*I fragment (designated pJCP802). The complete nucleotide sequence of this insert was determined. The region included an open reading frame sufficient to encode a polypeptide with an M_r of 107,751. An active hyaluronidase with an M_r of ≈89,000 was purified to homogeneity from *E. coli* DH5α(pJCP802). N-terminal amino acid sequence analysis of the purified protein suggested that translation initiation was occurring primarily at a TTG codon within the major open reading frame. However, immunoblot analysis using antiserum raised against the purified 89-kDa hyaluronidase indicated that *E. coli* DH5α(pJCP802) also expressed the 107-kDa form of the enzyme. This antiserum labelled a 107-kDa protein in partially purified hyaluronidase preparations from *S. pneumoniae*. The hyaluronidase activity in this pneumococcal extract was also neutralized by the antiserum.

Streptococcus pneumoniae is an important cause of life-threatening diseases such as pneumonia, bacteremia, and meningitis, as well as less severe but highly prevalent infections such as otitis media and sinusitis. Such infections are generally preceded by colonization of the nasopharyngeal mucosa (23), and in one study, it was estimated that approximately 15% of young children colonized by pneumococci went on to develop disease (8). Little, however, is understood of the mechanism whereby the colonizing pneumococci penetrate the physical defenses of the host and invade tissues (3).

We have previously shown that both pneumolysin and autolysin contribute to the virulence of *S. pneumoniae*. Pneumococci carrying defined mutations in either of these genes were less virulent than their otherwise isogenic parents (1, 2). However, the fact that these mutants were still capable of killing mice, albeit at high doses, suggested that other pneumococcal products were also important. It has been known for many years that pneumococci, as well as other members of the genus *Streptococcus*, produce hyaluronidase (11, 21). This enzyme hydrolyzes hyaluronic acid, which is an important component of connective tissues, and thus hyaluronidase might contribute directly to invasion. Boulnois et al. (4) have reported that the vast majority of clinical isolates of *S. pneumoniae* produce hyaluronidase. They also reported the isolation of a hyaluronidase-producing lambda gt11 clone from a pneumococcal gene library (4). Further characterization of this gene or of the hyaluronidase itself has not yet been reported.

In the present study we describe the cloning and sequencing of the gene encoding the pneumococcal hyaluronidase as well as a procedure for purification of the enzyme from recombinant *Escherichia coli*.

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MATERIALS AND METHODS

Bacterial strains and cloning vectors. The *S. pneumoniae* strains used in this study were clinical isolates belonging to serotypes 20 and 23. These organisms were routinely grown in Todd-Hewitt broth containing 0.5% yeast extract or on blood-agar plates. The *E. coli* K-12 strains, DH1 (9) and DH5α (Bethesda Research Laboratories, Gaithersburg, Md.), were grown in Luria-Bertani (LB) medium (18) with or without 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.). Where appropriate, ampicillin or kanamycin was added to growth media at a concentration of 50 μg/ml. The 10.2-kb, low-copy-number, inducible cosmid vector pOU61cos, which encodes ampicillin resistance, has been described by Knott et al. (15). The 2.4-kb plasmid pK184, which encodes kanamycin resistance, has also been described previously (13), and the 3.0-kb phagemid pBluescript SK, which encodes ampicillin resistance, was obtained from Stratagene, La Jolla, Calif.

***S. pneumoniae* chromosomal DNA extraction.** *S. pneumoniae* chromosomal DNA for use in cloning and Southern blot hybridization experiments was extracted and purified as described previously (24).

Construction of cosmid gene bank of *S. pneumoniae*. High-molecular-weight chromosomal DNA from the serotype 23 clinical isolate of *S. pneumoniae* was digested briefly with *Sau*3A1 so as to optimize the yield of fragments in the size range 35 to 40 kb. This DNA was ligated with a fivefold molar excess of pOU61cos DNA, which had been digested with *Bam*HI and *Xba*I. Double digestion of this double *cos* vector prevents recircularization of the cosmid and favors formation of packagable concatemers with pneumococcal DNA. Ligated DNA was packaged into lambda heads with a Packagene kit (Promega, Madison, Wis.) and transfected into *E. coli* DH1, which had been grown in LB medium containing 2% maltose. The cells were then plated onto LB agar supplemented with 50 μg of ampicillin per ml and incubated for 36 h at 30°C (at

which temperature the copy number of the cosmid remains low, as it is regulated by *c*I857 and lambda P_R). Cosmid clones were stored in LB medium containing ampicillin and 15% glycerol in microtiter plates at −70°C.

Hyaluronidase assay. The substrate for the hyaluronidase assay was human umbilical cord hyaluronic acid (Sigma Chemical Co., St. Louis, Mo.), dissolved in assay buffer (150 mM NaCl, 200 mM sodium acetate, pH 6.0) at a concentration of 1 mg/ml. Enzyme samples were diluted to 400 μl in assay buffer and incubated with 100 μl of substrate for 15 min at 37°C. The reaction was then stopped by the addition of 1 ml of 2% NaOH–2.5% cetrimide (Sigma), and the A_{400} was determined. Decrease in A_{400} with respect to a zero enzyme blank was linearly related to enzyme concentration over the range 2 to 10 NF units (NFU) per assay, with bovine testicular hyaluronidase (type IV-S; specific activity, 980 NFU/mg of protein) (Sigma) as the standard.

Preparation of crude pneumococcal hyaluronidase and antiserum. Approximately 30 clinical isolates of *S. pneumoniae* were screened for hyaluronidase production, and the strain producing the highest level (a type 20 strain) was used as the source of enzyme. This strain was grown overnight at 37°C in 54 liters of modified Trypticase soy broth (25), and cells were harvested with an Amicon DC10LA hollow-fiber concentrator fitted with an H5MP01 cartridge (0.1-μm cutoff). Cells were washed and resuspended in 10 mM sodium phosphate, pH 7.0, and lysed by treatment in a French pressure cell at 16,000 lb/in^2. Cell debris was removed by centrifugation at 35,000 × *g* for 20 min at 4°C, and the cleared lysate was loaded onto a column (5 by 60 cm) of DEAE Sepharose CL-6B (Pharmacia, Uppsala, Sweden). The column was eluted with a linear gradient of 10 to 250 mM sodium phosphate, pH 7.0. Fractions were assayed for hyaluronidase, and peak fractions were pooled, concentrated by ultrafiltration, and subjected to gel permeation chromatography on a column (2.5 by 100 cm) of Sephacryl S200-HR (Pharmacia), developed with 50 mM sodium phosphate, pH 7.0. Active fractions were again pooled and concentrated by ultrafiltration. The total preparation contained approximately 200 μg of protein, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicated that it consisted of four major protein species. The specific hyaluronidase activity of this impure preparation, however, was approximately 10 times that of the testicular hyaluronidase used as a standard in the assay.

This crude material was used to immunize three outbred, 8-week-old Quackenbush strain mice. These mice were injected intraperitoneally with 0.2-ml volumes of an emulsion (1:1) containing 10 μg of antigen in phosphate-buffered saline and Freund complete adjuvant (Commonwealth Serum Laboratories [CSL], Melbourne, Australia). At 14-day intervals, the mice were given two additional injections of antigen emulsified in Freund incomplete adjuvant (CSL). Blood samples were collected 7 days after the last injection. Sera were tested for the presence of antibodies by gel double immunodiffusion.

Screening for hyaluronidase-positive recombinants. Cosmid clones were grown overnight at 30°C in 200 μl of LB medium containing ampicillin in microtiter plates and then heat induced at 42°C for 2 h. The plates were centrifuged at 1,500 × *g* for 10 min, and cell pellets were suspended in 10 μl of 10 mM Tris-HCl–50 mM EDTA–10% sucrose–1 mg of lysozyme per ml (pH 8.0). After 1 h at 37°C, cells were lysed by the addition of 10 μl of 50 mM Tris-HCl–66 mM EDTA–0.4% Triton X-100. The plates were again centrifuged, and 3 μl of the supernatant from each well was spotted onto a nitrocellulose filter. Filters were then blocked as described by Towbin et al. (28) and reacted with the crude mouse anti-hyaluronidase serum, used at a dilution of 1:1,000. Filters were developed as described for Western blots (immunoblots), below.

Purification of hyaluronidase from recombinant *E. coli*. *E. coli* DH5α(pJCP802) was grown in 4 liters of LB medium containing ampicillin in a New Brunswick BioFlo IIc fermentor. Cells were harvested at the end of the logarithmic phase of growth (A_{600} of approximately 4.0), lysed, and chromatographed on DEAE Sepharose CL-6B and Sephacryl S200-HR, as described for the pneumococcal extract (above). The pooled post-Sephacryl hyaluronidase material was then diafiltered against 50 mM sodium phosphate (pH 7.0)–1.7 M ammonium sulfate, loaded onto a column (1.6 by 20 cm) of Phenyl Sepharose CL-4B (Pharmacia), and eluted with a 1-liter linear (descending) gradient of 1.7 M to 0 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0. Active fractions were pooled, concentrated, and stored at −15°C in 50 mM sodium phosphate (pH 7.0)–50% glycerol. The final yield of purified hyaluronidase was approximately 1 mg/liter of culture.

Protein assay. Protein concentrations were measured by the method of Bradford (5), with bovine serum albumin as the standard.

Southern blot analysis. DNA was digested with the appropriate restriction enzymes under the conditions recommended by the supplier. Digests were electrophoresed on 1.0% agarose gels with a Tris-borate-EDTA buffer system, as described by Maniatis et al. (18). DNA was transferred to nylon membranes (Hybond N$^+$; Amersham, Buckinghamshire, England) as described by Southern (27). DNA was fixed onto the filters by treatment with 0.4 M NaOH, prehybridized, and then hybridized to probe DNA and washed at high stringency, as described by Maniatis et al. (18). Probe DNA was labelled according to the method of Feinberg and Vogelstein (7) in the presence of digoxigenin-11-dUTP (Boehringer, Mannheim, Germany). Washed filters were developed with anti-digoxigenin–alkaline phosphatase conjugate (Boehringer) and a 4-nitroblue tetrazolium–X-phosphate substrate system, according to the manufacturer's instructions.

Plasmid DNA preparation. Plasmid DNA was prepared by the alkaline lysis miniprep method of Morelle (22).

Transformation. Transformation of *E. coli* with plasmid DNA was carried out with $CaCl_2$-treated cells as described by Brown et al. (6).

PAGE and Western blot analysis. SDS-PAGE was carried out as described by Laemmli (16), and where appropriate, gels were stained with Coomassie brilliant blue R250. For Western blot analysis, proteins were electrophoretically transferred from SDS-PAGE gels onto nitrocellulose filters, as described by Towbin et al. (28). Filters were probed with hyaluronidase antiserum or control serum (used at a dilution of 1:1,000) followed by goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Bio-Rad Laboratories, Richmond, Calif.). Enzyme-labelled bands were visualized with a 4-chloro-1-naphthol–H_2O_2 substrate system.

DNA sequencing. Nested deletions of cloned DNA in pBluescript SK were constructed by the method of Henikoff (10) with an Erase-a-Base kit (Promega). These were transformed into *E. coli* DH5α, and plasmid DNA was extracted from the resultant clones, as described above. Double-stranded-DNA sequencing was then carried out with dye-labelled primers on an Applied Biosystems model 373A automated DNA sequencer.

Amino acid sequencing. Sequencing was carried out on purified hyaluronidase, which had been electroblotted onto an Immobilon-P membrane, as described by Matsudaira (20). Protein bands were excised from the membrane, placed in a modified cartridge, as described by Williams et al. (29), and

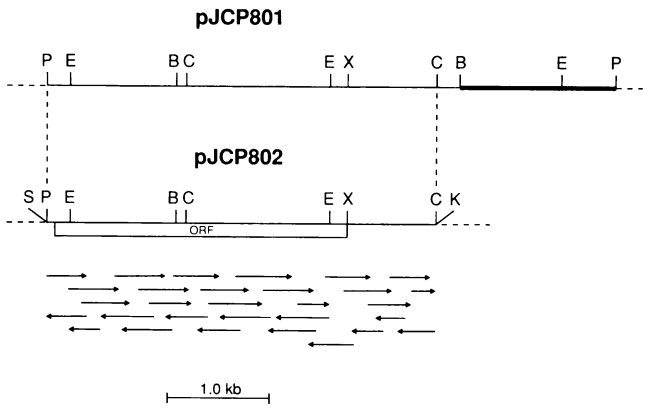


FIG. 1. Map of the inserts of pJCP801 and pJCP802 and scheme for sequencing. Restriction sites are indicated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sma*I; X, *Xba*I. Vector DNA (pK184 in pJCP801; pBluescript SK in pJCP802) is represented by the broken lines. The unbroken line denotes insert DNA, and the portion of the insert of pJCP801 derived from pOU61cos is shown in boldface type. The box labelled ORF denotes the position of the largest open reading frame in the insert of pJCP802. The arrows beneath the map indicate the portions of the plus (→) and minus (←) strands that were sequenced with nested-deletion derivatives of pJCP802 as templates.

analyzed on an ABI 470A protein sequenator equipped with an on-line phenylthiohydantoin-amino acid analyzer.

Nucleotide sequence accession number. The nucleotide sequence of the insert of pJCP802 has been deposited with GenBank (accession number L20670).

RESULTS

Isolation of hyaluronidase-producing clones from a cosmid gene bank and subcloning. A gene bank of *Sau*3A1-generated *S. pneumoniae* type 23 DNA fragments was constructed in *E. coli* DH1 with the low-copy-number cosmid vector pOU61cos, as described in Materials and Methods. Cell lysates were prepared from approximately 800 of the recombinant clones, and these were screened by immunoblotting using a mouse antiserum raised against a crude pneumococcal hyaluronidase preparation. One immunoreactive clone was detected, and lysates from this clone produced high levels of hyaluronidase activity after induction at 42°C for 2 h (approximately 39,000 NFU/ml). This clone contained a recombinant cosmid (designated pJCP800) with a 30- to 35-kb pneumococcal DNA insert.

In order to subclone the hyaluronidase coding region, pJCP800 DNA was digested with *Pst*I or *Hin*dIII and fragments were ligated with appropriately digested pK184 and transformed into *E. coli* DH5α. Lysates of kanamycin-resistant transformants were then assayed for hyaluronidase activity. A recombinant plasmid, designated pJCP801, with a 5.6-kb *Pst*I DNA insert was isolated from a hyaluronidase-positive clone. A restriction map of pJCP801 is shown in Fig. 1. The 5.6-kb insert of pJCP801 included approximately 1.5 kb derived from pOU61cos. To further localize the hyaluronidase gene within the insert of pJCP801, a deletion derivative lacking approximately 600 bp from the lefthand end of the pJCP801 insert (i.e., the end opposite that containing the cosmid-derived sequences) was constructed by exonuclease III digestion, and *E. coli* DH5α harboring this plasmid failed to produce hyaluronidase activity. This suggested that the coding region was located in the proximal portion of pJCP801, and so a 3.8-kb *Pst*I-*Cla*I fragment was subcloned into pBluescript SK (Fig. 1).

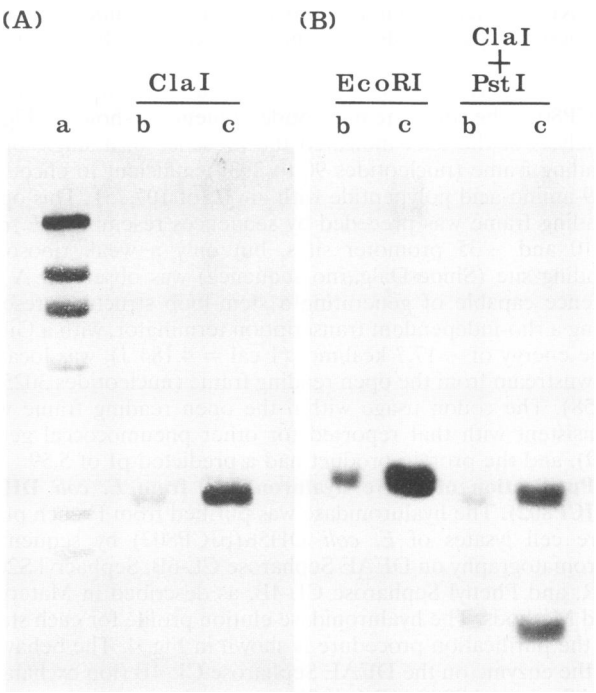


FIG. 2. Southern hybridization analysis. *S. pneumoniae* serotype 23 DNA (lanes b) and pJCP802 DNA (lanes c) were digested with the indicated restriction enzymes, transferred to nylon membranes, and hybridized with either the 1.4-kb *Cla*I-*Eco*RI fragment excised from the central portion of the insert of pJCP802 (A) or the 2.6-kb *Eco*RI fragment from pJCP802 (B). Probes were labelled with digoxigenin, and filters were developed as described in Materials and Methods. Lane a in panel A contains *Hin*dIII-digested lambda DNA which had been prelabelled with digoxigenin (fragment sizes 23.1, 9.4, 6.6, 4.37, 2.3, and 2.0 kb, respectively, from top to bottom).

Subcloning was carried out in two steps owing to the presence of an additional *Cla*I site approximately 1.4 kb from the lefthand end of the insert. This involved cloning the 1.4-kb *Pst*I-*Cla*I fragment into *Pst*I-*Cla*I-digested pBluescript SK, and then the 2.4-kb *Cla*I fragment was inserted into this construct after linearization with *Cla*I. The correct orientation of the *Cla*I fragment with respect to the proximal region was confirmed by restriction analysis using *Eco*RI and *Xba*I. *E. coli* DH5α carrying this plasmid, designated pJCP802, produced 1,560 NFU of hyaluronidase per ml of cell lysate.

To confirm that the pneumococcal DNA insert in pJCP802 had not undergone a major rearrangement during cloning and subcloning, plasmid DNA, as well as *S. pneumoniae* serotype 23 DNA, was digested with *Cla*I, *Eco*RI, or *Cla*I plus *Pst*I and subjected to Southern hybridization analysis (Fig. 2). On the basis of the restriction map of pJCP802 (Fig. 1), reactive fragment sizes of 2.4 kb, 2.5 kb, and 2.4 kb plus 1.4 kb would also be expected for the three respective digests of *S. pneumoniae* DNA. Both *S. pneumoniae* serotype 23 and pJCP802 digests contained probe-reactive restriction fragments of the expected size, indicating that the insert of pJCP802 represented contiguous pneumococcal DNA sequences. These data also suggested that there is only one copy of the hyaluronidase gene in the *S. pneumoniae* chromosome.

DNA sequence analysis. Nested-deletion derivatives of pJCP802 were constructed, and both strands of the insert were sequenced (see Fig. 1 for the sequencing scheme). The DNA sequence of appropriate nested-deletion derivatives of a 2.5-kb

*Eco*RI fragment from pJCP801, which was subcloned into pBluescript SK, was also determined to confirm that there was not a second internal *Cla*I site within the gene which might have resulted in a small deletion during construction of pJCP802. The complete nucleotide sequence is shown in Fig. 3. Analysis of the data indicated the presence of a single open reading frame (nucleotides 90 to 2939) sufficient to encode a 949-amino-acid polypeptide with an M_r of 107,751. This open reading frame was preceded by sequences resembling *E. coli* −10 and −35 promoter sites, but only a weak ribosome binding site (Shine-Dalgarno sequence) was observed. A sequence capable of generating a stem-loop structure, resembling a rho-independent transcription terminator, with a Gibbs free energy of −17.7 kcal/mol (1 cal = 4.184 J), was located downstream from the open reading frame (nucleotides 3025 to 3058). The codon usage within the open reading frame was consistent with that reported for other pneumococcal genes (19), and the protein product had a predicted pI of 5.59.

Purification of active hyaluronidase from *E. coli* DH5α (pJCP802). The hyaluronidase was purified from French pressure cell lysates of *E. coli* DH5α(pJCP802) by sequential chromatography on DEAE Sepharose CL-6B, Sephacryl S200-HR, and Phenyl Sepharose CL-4B, as described in Materials and Methods. The hyaluronidase elution profile for each stage of the purification procedure is shown in Fig. 4. The behavior of the enzyme on the DEAE Sepharose CL-4B (ion exchange) and Sephacryl S200-HR (gel filtration) columns was consistent with the pI and M_r predicted from the sequence data. SDS-PAGE analysis of pooled fractions at each step is shown in Fig. 5, and this indicated that the final purified hyaluronidase preparation consisted of a single protein species with an apparent M_r of approximately 89,000. This material had high hyaluronidase activity (764,000 NFU/mg of protein), which was approximately 800 times that of the commercial bovine testicular hyaluronidase preparation used as a standard.

N-terminal amino acid sequence and Western blot analysis. The apparent size of the pneumococcal hyaluronidase purified from recombinant *E. coli* (89 kDa) was smaller than that expected from the DNA sequence data (107 kDa). Possible reasons for this discrepancy include recognition of an alternative translation initiation codon and posttranslational proteolytic processing. To examine this, the amino acid sequence of the N terminus of the purified 89-kDa hyaluronidase was determined. The sequence obtained for the first 16 residues was M-K-I-L-A-S-V-K-D-T-Y-T-D-R-L-D. This sequence is identical to residues 164 to 179 of the predicted amino acid sequence for the 107-kDa polypeptide, except that the deduced sequence is L rather than M at position 164. This implies that initiation is occurring at the TTG codon at this position and that therefore the amino acid specified is M rather than L. Initiation at this point would be expected, from the DNA sequence data, to result in a 786-amino-acid polypeptide with an M_r of 89,413, which agrees closely with that of the purified hyaluronidase, as estimated by SDS-PAGE.

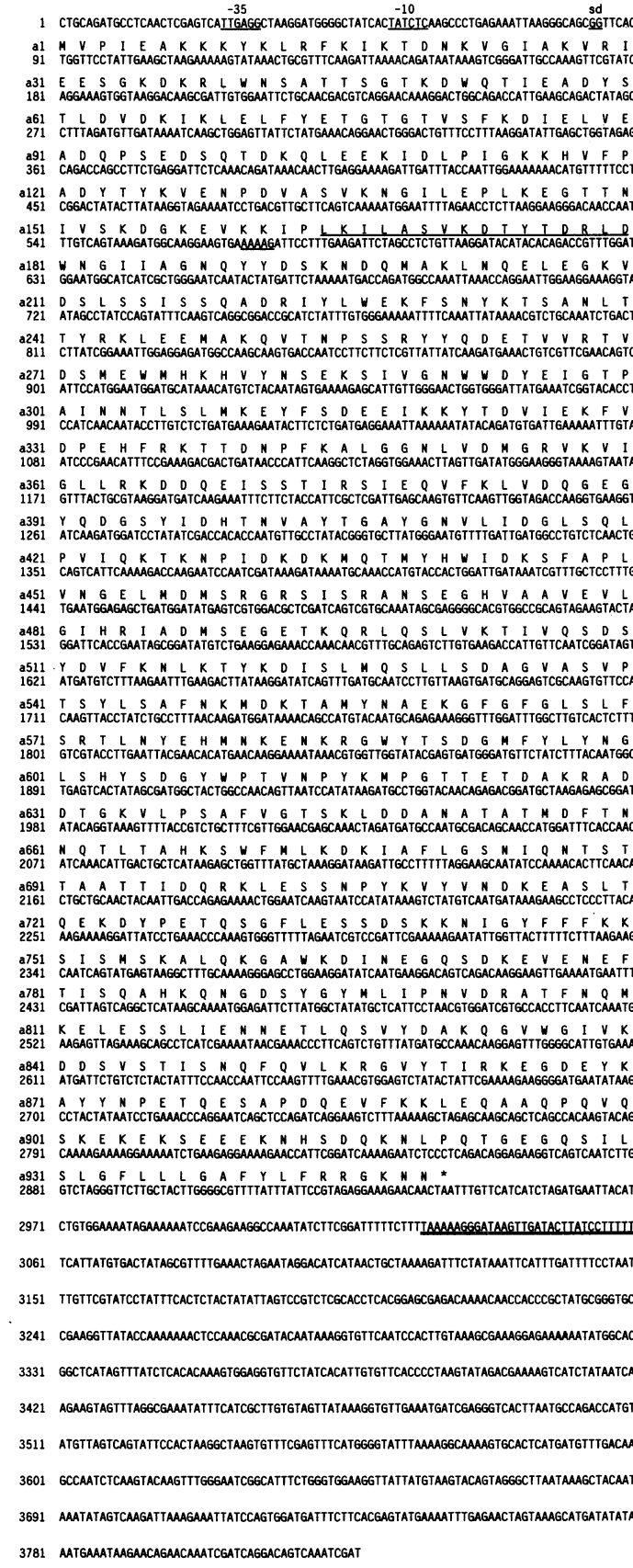
The hyaluronidase produced by *E. coli* DH5α(pJCP802) and *S. pneumoniae* was also analyzed, with a mouse antiserum raised against the purified recombinant hyaluronidase. The antiserum (but not preimmune mouse serum) completely neutralized hyaluronidase activity in extracts of *E. coli* DH5α(pJCP802) and a partially purified hyaluronidase extract from *S. pneumoniae* (results not shown). When these extracts were subjected to Western blot analysis (Fig. 6), the antiserum labelled at least 20 protein species in extracts of *E. coli* DH5α(pJCP802), only three of which were detected in extracts of DH5α(pBluescript SK). The largest antibody-reactive pJCP802-encoded polypeptide was approximately 107 kDa, which agrees closely with the size of the predicted translation product of the complete open reading frame. A polypeptide of similar size was labelled in the partially purified hyaluronidase extract from *S. pneumoniae*. These results suggest that in *E. coli* at least two translation initiation sites are recognized and that the polypeptides transcribed from these sites are susceptible to extensive proteolytic cleavage. In *S. pneumoniae*, however, only a 107-kDa hyaluronidase antibody-reactive polypeptide was detected, suggesting that there is only one translation initiation site recognized. Moreover, there was no evidence for proteolytic processing of hyaluronidase in *S. pneumoniae*.

To further examine the possibility that hyaluronidase translation products are susceptible to proteolytic cleavage in *E. coli*, purification of the enzyme from extracts of *E. coli* DH5α(pJCP802) was carried out, as described above, except that all column buffers were supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF] and 10 mM EDTA). The final purified material consisted of approximately equal amounts of three protein species, with M_rs of 89, 91, and 94 kDa. All three species reacted strongly with the hyaluronidase antiserum on Western blots (Fig. 7).

DISCUSSION

Given its substrate specificity, it is possible that hyaluronidase plays a role in pneumococcal pathogenesis by allowing greater microbial access to host tissue for colonization. It may also play a role in the migration of the organism between tissues, for example, translocation from the lung to the vascular system. Another way in which hyaluronidase might contribute to virulence or survival of pneumococci in the host is by scavenging potential carbon sources, such as glucose and glucuronic acid. Further analysis of the contribution (if any) of hyaluronidase to the pathogenesis of pneumococcal disease has been frustrated by the lack of basic information on the enzyme (it has not been purified previously) or the gene which encodes it. In the present study we have cloned and sequenced the *S. pneumoniae* gene encoding hyaluronidase and purified an active hyaluronidase from recombinant *E. coli*. N-terminal amino acid sequence analysis indicated that the 89-kDa hyaluronidase, which was purified to apparent homogeneity as judged by SDS-PAGE, was likely to have been translated from an internal (in-frame) TTG codon within the major open reading frame. Western blot analysis using an antiserum raised against the 89-kDa enzyme specifically labelled a large number of polypeptides in extracts of *E. coli* DH5α(pJCP802), while there was negligible labelling of similar extracts derived from the parental host/vector. The largest immunoreactive species detected in the hyaluronidase-producing clone was ap-

FIG. 3. Nucleotide sequence of the insert of pJCP802. The amino acid translation (represented in the single-letter code) for the open reading frame (nucleotides 90 to 2939) is shown above the middle nucleotide of each codon. A possible ribosome binding site (sd) and putative −10 and −35 promoter regions upstream from the open reading frame are underlined. The portion of the deduced amino acid sequence corresponding to the N terminus of the 89-kDa polypeptide is also underlined, as is a possible alternative ribosome binding site upstream from this region. A possible transcription terminator sequence (nucleotides 3025 to 3058) is shown with a heavy underline.


-35
-10
sd

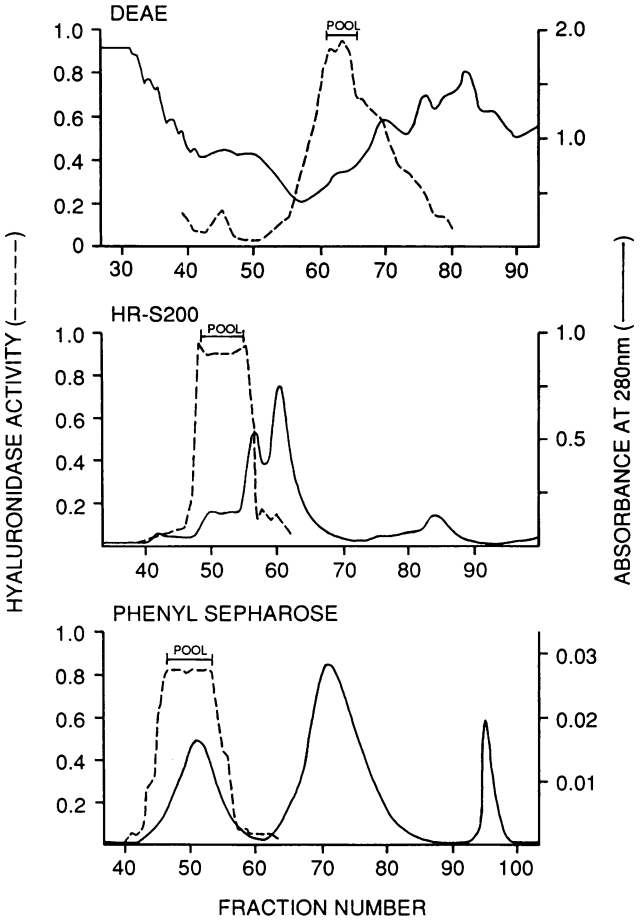


FIG. 4. Chromatographic purification of hyaluronidase. Column chromatography profiles for each stage of the purification procedure are shown. Hyaluronidase activity is expressed in arbitrary units. Horizontal bars indicate those fractions pooled prior to the next step.

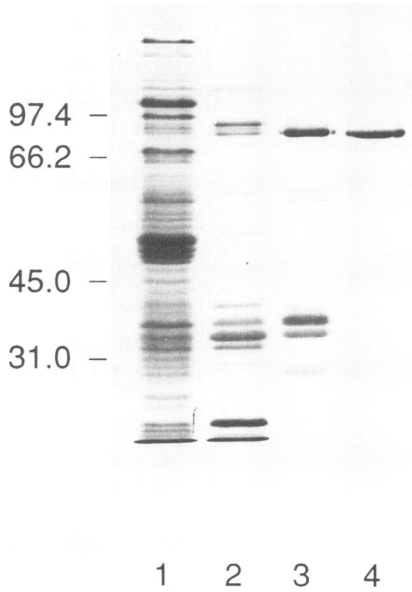


FIG. 5. SDS-PAGE analysis of purified hyaluronidase. Pooled fractions from the various stages of the purification procedure were electrophoresed on SDS–10% polyacrylamide gels and stained with Coomassie brilliant blue R250. The mobilities of various molecular size markers (expressed in kilodaltons) are indicated. Lanes: 1, crude *E. coli* DH5α(pJCP802) lysate; 2, post-DEAE Sepharose; 3, post-Sephacryl S200-HR; 4, post-Phenyl Sepharose (final purified material; approximately 5 μg of protein loaded).

proximately 107 kDa, the same size as that expected from the deduced amino acid sequence of the major open reading frame. Moreover, a 107-kDa protein was labelled in a partially purified hyaluronidase preparation from *S. pneumoniae*, and enzymatic activity in this extract was neutralized by the antiserum. The large number of smaller hyaluronidase antibody-reactive species in DH5α(pJCP802) extracts suggests that extensive proteolytic cleavage of hyaluronidase occurs in *E. coli*. Indeed, when protease inhibitors (PMSF and EDTA) were included in buffers used for purification of hyaluronidase from DH5α(pJCP802) lysates, the final preparation consisted of approximately equal amounts of 89-, 91-, and 94-kDa proteins, all of which reacted strongly with the hyaluronidase antiserum in Western blots. However, the fact that the N-terminal amino acid sequence of the 89-kDa form commenced with a TTG-encoded Met residue indicates that this species has not been subjected to N-terminal processing. There were no alternative potential initiation codons upstream which might account for the 91- and 94-kDa species, and so presumably these are intermediate degradation products of the 107-kDa primary translation product. There was insufficient resolution on SDS-PAGE to permit separate determination of the N-terminal amino acid sequence of the 91- and 94-kDa forms, and it is not known whether these particular forms are enzymatically active.

Collectively, the results described above imply that, in *E. coli*, the pneumococcal hyaluronidase is translated from at least two initiation codons and that these translation products are susceptible to proteolysis. It is also likely that two separate promoter sites are recognized, as *E. coli* carrying a deletion derivative of pJCP802 lacking nucleotides 1 to 329 retained full hyaluronidase activity. The deleted region includes the putative promoter site for the 107-kDa enzyme as well as the first 240 nucleotides of the major open reading frame. Thus, sequences downstream from nucleotide 330 are capable of directing expression of an active portion of the hyaluronidase coding sequence. Deletion derivatives lacking nucleotides 1 to 654 were hyaluronidase negative (results not presented). We are currently using promoterless chloramphenicol acetyltransferase expression vectors to locate promoter sequences in the region immediately upstream from the initiation codon (nucleotide 579) for the 89-kDa form of the enzyme. In *S. pneumoniae*, however, only one initiation site appears to be recognized and there was no evidence of proteolytic cleavage.

Regardless of the translation initiation site recognized in *E. coli*, the pneumococcal hyaluronidase lacks a signal peptide, and in *E. coli*, the enzyme remains cell associated. In *S. pneumoniae* cultures, hyaluronidase is found in both the culture supernatant and the cell-associated fraction. However, the appearance of hyaluronidase activity in the culture supernatant precisely parallels the appearance of pneumolysin (results not presented). Pneumolysin is a cytoplasmic protein which is released only when pneumococci undergo autolysis (1, 14). Thus, it appears that pneumococcal hyaluronidase may also be a cytoplasmic protein, a fact consistent with the absence of an N-terminal signal peptide.

The hyaluronidase gene from *Streptococcus pyogenes* bacteriophage H448A has also been cloned and sequenced (12). The

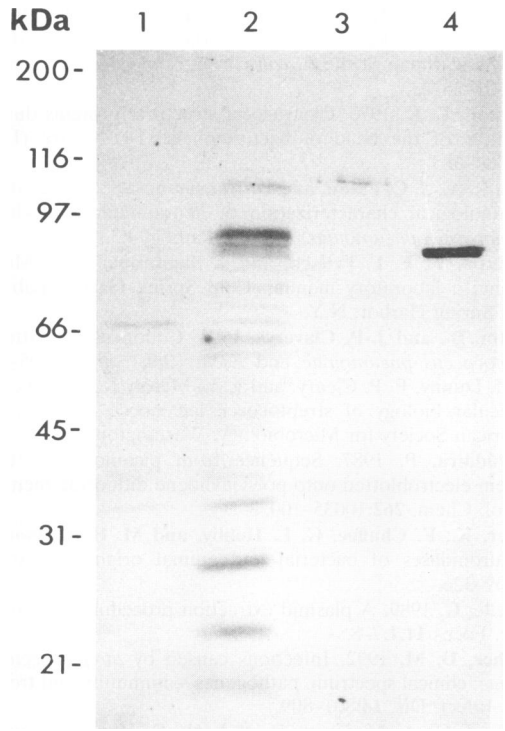


FIG. 6. Western blot analysis. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and reacted with mouse antiserum raised against the purified 89-kDa hyaluronidase, as described in Materials and Methods. Lanes: 1, *E. coli* DH5α(pBluescript SK) lysate; 2, *E. coli* DH5α(pJCP802) lysate; 3, hyaluronidase partially purified from *S. pneumoniae* type 20 (post-DEAE Sepharose chromatography); 4, purified 89-kDa hyaluronidase.

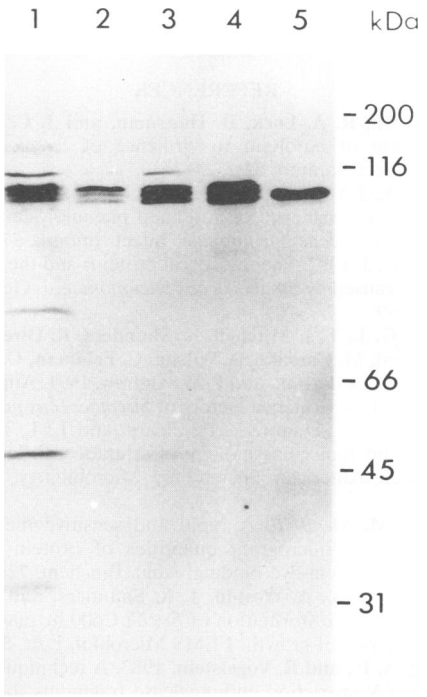


FIG. 7. Western blot analysis of pooled fractions from the various stages of the hyaluronidase purification procedure carried out in the presence of PMSF and EDTA. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and reacted with mouse antiserum raised against the purified 89-kDa hyaluronidase, as described in Materials and Methods. The mobilities of various molecular size markers (expressed in kilodaltons) are indicated. Lanes: 1, crude *E. coli* DH5α(pJCP802) lysate; 2, post-DEAE Sepharose; 3, post-Sephacryl S200-HR; 4, post-Phenyl Sepharose; 5, 89-kDa hyaluronidase marker (purified in the absence of protease inhibitors).

encoded protein is much smaller (39.5 kDa) than the pneumococcal enzyme, and there is negligible homology at the amino acid level between the two sequences. An apparently unpublished sequence for the hyaluronidase gene of *Clostridium perfringens* has also been deposited in GenBank (accession number M81878). The size of this enzyme, calculated from the deduced amino acid sequence (117.6 kDa), is closer to that of the pneumococcal hyaluronidase, but again there is no significant homology between the two enzymes at the amino acid level. Thus, sequence comparisons have not identified any structurally conserved regions, which may have provided an indication of residues important for catalytic activity. Clearly, for the pneumococcal hyaluronidase, the N-terminal region is not essential, as the 89-kDa form, which lacks the first 163 residues found in the 107-kDa form, retains high specific activity (800 times that of a commercial bovine testicular hyaluronidase preparation). The 107-kDa form of hyaluronidase is also presumed to be enzymatically active, as no other species were detected in pneumococcal lysates. In this respect, hyaluronidase is remarkably similar to the pneumococcal neuraminidase; multiple forms of this enzyme also appear to exist, the largest being 107 kDa. However, proteolytic cleavage generates an 86-kDa fragment which retains full neuraminidase activity (17). Interestingly, it has been reported recently that the neuraminidase of group B streptococci is actually a hyaluronidase (26), raising the possibility that, in *S. pneumoniae*, the two enzymes might actually be the same protein. However, the purified pneumococcal hyaluronidase had no detectable neuraminidase activity when 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid was used as the substrate, and purified pneumococcal neuraminidase did not have any hyaluronidase activity. Furthermore, there was no immunological cross-reaction between the two proteins (results not presented).

Western blot analysis suggested that the total amount (by weight) of enzyme produced in *S. pneumoniae* may be very low, as only weak signals were obtained when crude pneumococcal culture lysates were probed (results not shown). Indeed, it was necessary to partially purify and concentrate these lysates in order to obtain a signal strong enough for photographic reproduction. The apparent differences in intensity of labelling in Western blots were nevertheless consistent with the total amounts of enzyme activity present in *S. pneumoniae* and recombinant *E. coli* lysates.

Some uncertainty remains concerning the precise regulatory regions involved in expression of hyaluronidase both in *E. coli* and in *S. pneumoniae*. Nevertheless, access to the cloned gene and determination of the complete nucleotide sequence will allow the study of the role of hyaluronidase in vivo via the construction of defined hyaluronidase-negative pneumococci for use in animal models of infection.

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