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# *Streptococcus pneumoniae* Produces at Least Two Distinct Enzymes with Neuraminidase Activity: Cloning and Expression of a Second Neuraminidase Gene in *Escherichia coli*

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**A gene from *Streptococcus pneumoniae* was cloned in lambda EMBL301 and then expressed in *Escherichia coli*, which cleaved the fluorogenic neuraminidase substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid. The cloned gene therefore encodes an enzyme with neuraminidase activity. On the basis of restriction mapping and DNA hybridization studies, this gene could be distinguished from another pneumococcal neuraminidase gene cloned previously (A. M. Berry, J. C. Paton, E. M. Glare, D. Hansman, and D. E. A. Catcheside, Gene 71:299–305, 1988). Both neuraminidase genes were found in each of five isolates, covering at least three serotypes, of pneumococci tested.**

*Streptococcus pneumoniae* is an important human pathogen responsible for lower respiratory tract infections, septicemia, and meningitis. It produces several factors which are of possible relevance in the pathogenesis of disease. One of these is neuraminidase, an enzyme which cleaves *N*-acetylneuraminic acid (NeuNAc) from mucin, glycoproteins, and gangliosides (8, 17). Although the role of neuraminidase is unclear, there is some indirect evidence to suggest that it may function as a pneumococcal virulence factor. For example, all clinical isolates produce neuraminidase (8, 14), and intracerebral inoculation of mice with partially purified neuraminidase caused neurological disease signs and death (7). However, these symptoms were not apparent on intrathecal inoculation of the enzyme in dogs (15). Mice immunized with purified neuraminidase were partially protected from pneumococcal infection (12). In some patients with pneumococcal infection, there is a relationship between the concentration of NeuNAc in cerebrospinal fluid and coma and adverse outcome (14). While the precise role that neuraminidase plays is unclear, it has been suggested that release of NeuNAc from gangliosides may contribute to morbidity and mortality of pneumococcal meningitis (14) and may also expose receptors for pneumococcal attachment to cells (1).

There are conflicting views regarding the number of neuraminidase enzymes produced by the pneumococcus. Several pneumococcal neuraminidase isoenzymes of about 70 kDa have been described, corresponding to four groups of neuraminidases in terms of charge (21, 22). Other reports ascribe molecular masses for pneumococcal neuraminidase of between 65 and 107 kDa (2, 11, 17, 20). However, both the disparities in the reported molecular weights and the apparent existence of isoenzyme forms have been postulated to be a consequence of the susceptibility of a single neuraminidase enzyme to proteolytic cleavage (11). To explore the production of neuraminidase by the pneumococcus and to facilitate studies directed towards defining a role (if any) of the enzyme in pneumococcal infection, we sought to clone the pneumococcal gene for neuraminidase. Here, we describe the cloning in *Escherichia coli* of a pneumococcal gene which expresses a product with neuraminidase activity. This gene is distinct from a pneumococcal neuraminidase gene cloned previously (2), and both genes were present in each of five pneumococcus isolates tested.

A gene bank of pneumococcal strain R36A (NCTC 10319), a strain derived from a type II isolate, was constructed in the lambda vector EMBL301. *S. pneumoniae* chromosomal DNA, prepared as described previously (23), was partially digested with *Sau*3AI to generate fragments of lengths greater than 20 kb. Lambda EMBL301 (10) was purified and arms were prepared as previously described (3). Ligation of vector and insert DNA was done without prior size fractionation of the digested chromosomal DNA. After in vitro packaging, phage were plated on *E. coli* Q359 (6) as described previously (3).

A total of 800 nonamplified recombinant plaques were screened for neuraminidase activity by using an agar overlay containing the fluorogenic neuraminidase substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN; 100 μg/ml) (Sigma) in 0.75% agar dissolved in a solution containing sodium acetate (50 mM, pH 5.5), NaCl (150 mM), and $CaCl_2$ (4 mM). After 1 h at 37°C, the plates were observed under UV light (wavelength, 366 nm), and positive clones were detected by their associated fluorescence resulting from the release of 4-methylumbelliferone from MUAN. One recombinant expressing neuraminidase activity was found. This recombinant (EMBL301-neu1) had an insert of 18.5 kb of DNA. Purified lambda EMBL301-neu1 DNA hybridized in dot blots to DNA from pneumococcal strains R36A, DP1601 (a derivative of a type II isolate [see reference 18] from J. P. Claverys, Toulouse, France), and GB05 (a type III pneumococcal clinical isolate) (data not shown), and hence contained pneumococcal DNA.

DNA from the lambda recombinant EMBL301-neu1 was partially digested with *Sau*3AI, the products were size fractionated in gels (9), and 4- to 5-kb fragments were cloned into *Bam*HI-cleaved pJDC9 (4). The pJDC9 vector was used to facilitate the stable cloning of pneumococcal DNA (4). Recombinant DNA was transformed into JM101 (24), selecting for resistance to erythromycin (1 mg/ml). Among the resulting clones with neuraminidase activity detected as previously described (2), the smallest plasmid found

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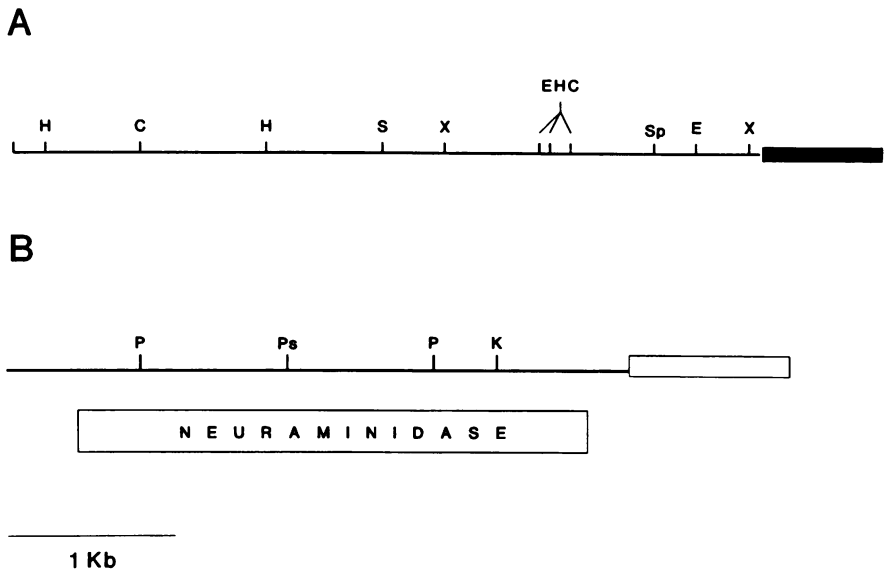


FIG. 1. Restriction map of cloned pneumococcal DNA in pMC2150 (A) and pJCP301 (B) (2). Restriction endonuclease sites: H, *Hin*dIII; C, *Cla*I; S, *Sac*I; X, *Xba*I; E, *Eco*RV; Sp, *Sph*I; P, *Pvu*II; Ps, *Pst*I; K, *Kpn*I. Note that the order of the E, H, and C sites grouped in the center of the pMC2150 insert is not known. Several *Eco*RI and *Bam*HI sites were present but not mapped. No sites for *Sma*I, *Kpn*I, *Pst*I, or *Sal*I were present in the insert in pMC2150. Berry et al. (2) found no sites in pJCP301 for *Cla*I, *Eco*RI, *Bam*HI, *Hin*dIII, or *Xba*I. Closed and open boxes indicate the vectors pJDC9 (A) and pHC79 (B), respectively. The likely extent of the neuraminidase gene in pJCP301 (2) is indicated.

(pMC2150) had an insert size of 4.3 kb. A restriction map of this insert was constructed by performing double and triple enzyme digests (Fig. 1). Comparison of the restriction map of pMC2150 with that of pJCP301, the plasmid carrying the pneumococcal neuraminidase gene cloned by Berry et al. (2), revealed no obvious similarity (Fig. 1).

To determine whether there was DNA sequence homology between the neuraminidase gene cloned from a type I strain by Berry et al. (2) and carried on pJCP301 and that present in pMC2150 from a type II strain, a Southern blot analysis (19) was performed. DNA was digested with a variety of enzymes, and the resulting DNA fragments were electrophoresed in 1% agarose gels and transferred to Hybond N filters (Amersham) as previously described (13). Probe DNA was prepared (5) and hybridization was done (13) as previously described. The final filter wash was done at 65°C in either 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate)–0.1% sodium dodecyl sulfate (SDS) or 0.2× SSC–0.1% SDS. Various digests of pJCP301 were probed with the 4.3-kb insert of pMC2150, isolated as a *Kpn*I-*Pst*I fragment by digestion within the polylinker of pJDC9. No hybridization was found when the filters were washed at 65°C at low (2× SSC) or high (0.2× SSC) stringency (data not shown). Thus, the sequences present in pJCP301 and pMC2150 are clearly distinct.

To assess whether both cloned sequences are present in individual pneumococcal isolates, chromosomal DNA from pneumococcal strains R36A, DP1601, and GB05 was cleaved with either *Cla*I (Fig. 2A) or *Pvu*II (Fig. 2B) and probed with inserts from pMC2150 (isolated as above) and pJCP301 (isolated as a 1.6-kb *Pvu*II fragment), respectively. *Cla*I fragments of 3.0 and 2.45 kb hybridized in R36A and DP1601, while in GB05, a 5.5-kb *Cla*I fragment hybridized to the pMC2150-derived probe (Fig. 2A). Given the restriction map of the insert present in pMC2150, which contains two sites for *Cla*I, it was expected that three *Cla*I fragments would hybridize in the R36A genome. Only two were apparent, even though fragments of less than about 500 bp would have been detected. It is possible that one of the observed bands is a doublet. In contrast, and as expected, a single 1.6-kb *Pvu*II fragment in R36A, DP1601, and GB05 hybridized to the pJCP301-derived probe. Thus, the sequences cloned in pJCP301 and pMC2150 are present in each strain. In agreement with this, when DNA from the above three pneumococcal strains was cleaved with either *Hin*dIII or *Eco*RV and probed with each of the probes described above, different fragments within each genome bound the different probes (data not shown). Examination of DNA by dot blotting indicated that another three clinical isolates (a type I strain, designated 3551 [see reference 16] and two clinical

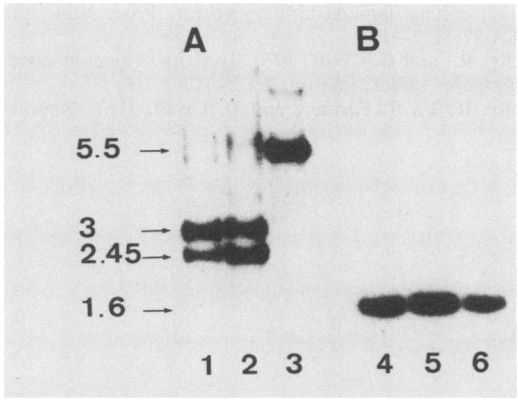


FIG. 2. Southern blot analysis of neuraminidase genes. (A) The 4.3-kb insert from pMC2150 was used to probe *Cla*I digests of chromosomal DNAs from R36A (lane 1), DP1601 (lane 2), and GB05 (lane 3). (B) An internal *Pvu*II fragment of pJCP301 (Fig. 1) was used to probe *Pvu*II-digested chromosomal DNAs from R36A (lane 4), DP1601 (lane 5), and GB05 (lane 6).

isolates chosen at random and of unknown serotype) of the pneumococcus carried both genes.

We have cloned a gene from *S. pneumoniae* which expresses a product which can cleave MUAN. Thus, the product has neuraminidase activity, although it is not yet known whether the enzyme can use other substrates. On the basis of restriction mapping and DNA hybridization studies, we conclude that the gene present on lambda EMBL01-neu1 and the previously cloned neuraminidase gene (2) are different. The genomes of the individual pneumococcal isolates thus far analyzed have both genes, although we do not know whether an individual strain expresses both genes simultaneously. The molecular relationship of the products of these two genes awaits the generation of the complete DNA sequence of each gene and/or the purification and characterization of the recombinant products. It is clear that the pneumococcus has the capacity to produce more than one product with neuraminidase activity, indicating that the presence of multiple enzymes with neuraminidase activity may not be only a consequence of the proteolytic degradation of a single neuraminidase.

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