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Cloning and Characterization of *nanB*, a Second *Streptococcus pneumoniae* Neuraminidase Gene, and Purification of the NanB Enzyme from Recombinant *Escherichia coli*

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Streptococcus pneumoniae **is believed to produce more than one form of neuraminidase, but there has been uncertainty as to whether this is due to posttranslational modification of a single gene product or the existence of more than one neuraminidase-encoding gene. Only one stable pneumococcal neuraminidase gene (designated *nanA*) has been described. In the present study, we isolated and characterized a second neuraminidase gene (designated *nanB*), which is located close to *nanA* on the pneumococcal chromosome (approximately 4.5 kb downstream). *nanB* was located on an operon separate from that of *nanA*, which includes at least five other open reading frames. NanB has a predicted size of 74.5 kDa after cleavage of a 29-amino-acid signal peptide. There was negligible amino acid homology between NanA and NanB, but NanB did exhibit limited homology with the sialidase of *Clostridium septicum*. NanB was purified from recombinant *Escherichia coli* and found to have a pH optimum of 4.5, compared with 6.5 to 7.0 for NanA. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis suggested that NanB has a molecular size of approximately 65 kDa. The discrepancy between this estimate and the size predicted from the nucleotide sequence is most likely a consequence of C-terminal processing or anomalous electrophoretic behavior.**

Streptococcus pneumoniae remains one of the foremost bacterial pathogens for humans, although the precise molecular mechanisms whereby it invades and damages its host are poorly understood. It has been known for many years that production of an antiphagocytic capsule is a sine qua non of virulence (1), but the capsular polysaccharides themselves are completely nontoxic and cannot account for death directly. Accordingly, much recent attention has focused on the possible contribution of pneumococcal proteins to the pathogenesis of disease (28). Studies using site-specific mutagenesis techniques have directly confirmed the involvement of pneumolysin (2, 6, 7), autolysin (3, 6), pneumococcal surface protein A (PspA) (24), and, most recently, pneumococcal surface adhesin A (PsaA) (4) in pathogenesis. However, evidence for the involvement of the pneumococcal neuraminidase is more indirect (28).

Neuraminidase cleaves terminal sialic acid residues from a wide variety of glycolipids, glycoproteins, and oligosaccharides on cell surfaces or in body fluids, and such activity has the potential to cause great damage to the host. Neuraminidase might also unmask potential cell surface receptors for putative pneumococcal adhesins (18). At least two studies on fresh clinical isolates of *S. pneumoniae* showed that all of the strains examined (104 in all) had neuraminidase activity (15, 26), and this is consistent with our own unpublished findings. Histochemical studies of organs from mice dying after intraperitoneal administration of partially purified pneumococcal neuraminidase have indicated marked decreases in the sialic acid contents of the kidneys and liver compared with those of controls (16). It has also been shown that both coma and bacteremia occur significantly more often among patients with pneumococcal meningitis when the concentration of *N*-acetylneuraminic acid in the cerebrospinal fluid is elevated (26).

Further assessment of the contribution made by neuraminidase to pneumococcal pathogenicity has been complicated by the fact that there appear to be multiple forms of the enzyme. Early studies suggested that pneumococcal neuraminidase exists as multiple isoenzymes with sizes of approximately 70 kDa (35–37), but we have previously suggested that these are a consequence of proteolytic degradation of a parental enzyme. We purified a single 107-kDa neuraminidase species from *S. pneumoniae* lysates treated with protease inhibitors, but in the absence of these, several smaller fully active forms (with molecular masses as low as 86 kDa) were isolated (20). Scanlon et al. (32) subsequently reported the purification of a 65-kDa pneumococcal neuraminidase, but this was prepared in the absence of protease inhibitors. We have also demonstrated that immunization of mice with the purified 107-kDa neuraminidase confers a limited degree of protection against challenge with virulent pneumococci, further suggesting a role for the enzyme in pathogenesis (21).

There have been reports of the cloning of two *S. pneumoniae* genes encoding neuraminidase into *Escherichia coli* (5, 11), both of which were isolated on the basis of the ability to cleave a synthetic fluorigenic neuraminidase substrate. Interestingly, hybridization analysis indicated that these two neuraminidase genes are different and that individual pneumococcal isolates contain both genes (11), a fact which has complicated construction of defined neuraminidase-negative pneumococci for virulence studies. The former neuraminidase-producing clone was unstable and could not be further characterized, but the neuraminidase-encoding gene in the latter clone (designated *nanA*) has been sequenced (10). While there is some ambiguity as to the precise translation initiation site for *nanA* (there are two in-frame ATG start codons preceded by Shine-Dalgarno sequences), the most likely primary translation product would

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have a size of 112 kDa, including a hydrophobic signal peptide at its N terminus (10). Cleavage of the signal peptide would yield a processed NanA of approximately 108 kDa, which is very close to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-based size estimate for the purified pneumococcal neuraminidase (107 kDa) which we have reported previously (20). Moreover, we have since subjected our purified enzyme to N-terminal amino acid sequence analysis and found the first 10 residues to be identical to those predicted for NanA after cleavage of the signal peptide (19a). In the present study, we found a molecular explanation for the conflicting data concerning the number and size of pneumococcal neuraminidases by cloning and sequencing a second pneumococcal neuraminidase gene which we have designated *nanB*. We also purified the product of this gene (NanB) and showed that it is much smaller than NanA, with a size of approximately 70 kDa.

MATERIALS AND METHODS

Bacterial strains and cloning vectors. The *S. pneumoniae* strain used in this study was a clinical isolate from the Women's and Children's Hospital belonging to serotype 6. This organism was routinely grown in Todd-Hewitt broth–0.5% yeast extract or on blood agar plates. *E. coli* K-12 strain DH1 (13) and strain DH5α (Bethesda Research Laboratories) were grown in Luria-Bertani medium (LB) (22) with or without 1.5% Bacto Agar (Difco Laboratories, Detroit, Mich.). When appropriate, ampicillin 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. (17). The 3.0-kb phagemid pBluescript SK, which encodes ampicillin resistance, was obtained from Stratagene, La Jolla, Calif. Plasmid pMC4170, which encodes *nanA* (10), was provided by T. Mitchell and P. Andrew.

Neuraminidase assay. Neuraminidase was assayed by using the fluorigenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN) (Sigma Chemical Co., St. Louis, Mo.) as previously described (20). One unit is defined as that amount which releases 1 μmol of 4-methylumbelliferone from MUAN in 1 min at 37°C.

***S. pneumoniae* chromosomal DNA extraction.** *S. pneumoniae* chromosomal DNA for use in cloning and Southern blot hybridization experiments was extracted and purified as previously described (27).

Construction of cosmid gene bank of *S. pneumoniae*. High-molecular-weight chromosomal DNA from the serotype 6 clinical isolate of *S. pneumoniae* was digested briefly with *Sau*3A1 to optimize the yield of fragments in the size range of 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 cosmid recircularization and favors formation of packagable concatemers with pneumococcal DNA. Ligated DNA was packaged into lambda heads with a Packagene kit (Promega Biotec, Madison, Wis.) and transfected into *E. coli* DH1 which had been grown in LB–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 cI857 and lambda P_R). Cosmid clones were stored in LB–ampicillin–15% glycerol in microtiter plates at −70°C.

Screening of cosmid clones by immunoblotting. Cosmid clones were grown overnight at 30°C in 200 μl of LB–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 resuspended 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 addition of 10 μl of 50 mM Tris-HCl, 66 mM EDTA, and 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. (38) and reacted with mouse antineuraminidase serum (20) at a dilution of 1:1,000. Filters were developed as described for Western blots (immunoblots) below.

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. (22). DNA was transferred to nylon membranes (Hybond N^+; Amersham, Buckinghamshire, England) as described by Southern (34). 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. (22). Probe DNA was labeled by the method of Feinberg and Vogelstein (12) in the presence of digoxigenin-11-dUTP (Boehringer, Mannheim, Germany). Washed filters were developed with an antidigoxigenin Fab fragment-alkaline phosphatase conjugate and a 4-nitroblue tetrazolium salt–5-bromo-4-chloro-3-indolylphosphate substrate system (Boehringer) in accordance with the manufacturer's instructions.

Plasmid DNA preparation. Plasmid DNA was prepared by the alkaline lysis miniprep method of Morelle (25).

Transformation. Transformation of *E. coli* with plasmid DNA was carried out with $CaCl_2$-treated cells as described by Brown et al. (9).

DNA sequencing. Nested deletions of pneumococcal DNA cloned into pBluescriptSK were constructed by the method of Henikoff (14) with an Erase-a-Base kit (Promega). This DNA was transformed into *E. coli* DH5α, and the resulting plasmid DNA was characterized by restriction analysis. Double-stranded template DNA for sequencing was prepared as recommended in the Applied Biosystems sequencing manual. The sequence of both strands was then determined by using dye-labelled primers on an Applied Biosystems 373A automated DNA sequencer. The sequence was analyzed with DNASIS and PROSIS Version 7.0 software (Hitachi Software Engineering, San Bruno, Calif.).

Purification of NanB from recombinant *E. coli*. *E. coli* DH5α(pJCP311) was grown in 4 liters of LB-ampicillin in a New Brunswick BioFlo IIc fermentor. Cells were harvested by centrifugation (15,000 × *g*, 10 min, 4°C) at the end of the logarithmic phase of growth (A_{600}, approximately 4.0) and resuspended in approximately 150 ml of 10 mM sodium phosphate, pH 7.0. Cells were lysed by treatment in an Aminco French pressure cell at 12,000 lb/in^2, and cellular debris was removed by centrifugation (100,000 × *g*, 60 min, 4°C). Neuraminidase was then purified by chromatography on DEAE-Sepharose CL-6B, Sephacryl S200-HR (Pharmacia Biotech, Uppsala, Sweden), and Amicon Red-A dye-ligand resin (Australasian Medical and Scientific, Chatswood West, New South Wales, Australia) by using the same buffers and elution conditions described previously for NanA (20). 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 NanB was approximately 6 mg/liter of culture.

Protein assay. Protein concentrations were measured by the method of Bradford (8) with bovine serum albumin as the standard.

SDS-PAGE and Western blot analysis. SDS-PAGE was carried out as described by Laemmli (19), and when appropriate, gels were stained with Coomassie brilliant blue R-250. For Western blot analysis, proteins were electrophoretically transferred from SDS-PAGE gels onto nitrocellulose filters as described by Towbin et al. (38). Filters were probed with mouse anti-NanA, anti-NanB or control serum (at a dilution of 1:1,000), followed by goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Bio-Rad Laboratories, Richmond, Calif.). Enzyme-labeled bands were visualized with a 4-nitroblue tetrazolium salt–5-bromo-4-chloro-3-indolylphosphate substrate system (Boehringer).

Amino acid sequencing. Sequencing was done with purified NanB which had been electroblotted onto an Immobilon P membrane as described by Matsudaira (23). Protein bands were excised from the membrane, placed in a modified cartridge as described by Williams et al. (39), and analyzed on an ABI 470A protein sequenator equipped with an on-line phenylthiohydantoin-amino acid analyzer.

Nucleotide sequence accession number. The nucleotide sequence described in this report has been deposited with GenBank under accession number U43526.

RESULTS

Cloning of a second pneumococcal neuraminidase gene. As part of ongoing studies in our laboratory on the genetic basis for the apparent diversity of pneumococcal neuraminidase(s), we constructed a cosmid gene bank of *S. pneumoniae* serotype 6 DNA in *E. coli* DH1 by using low-copy-number vector pOU61cos as described in Materials and Methods. This library, consisting of approximately 600 independent clones, was initially screened by colony immunoblotting using mouse anti-NanA serum as the probe. This resulted in the isolation of a single cosmid clone, the lysate of which also contained a high level of neuraminidase activity, as judged by its capacity to cleave the synthetic fluorigenic substrate MUAN. The recombinant cosmid from this clone (designated pJCP309) was purified, and restriction analysis indicated that it contained a pneumococcal DNA insert of approximately 32 kb. To isolate smaller DNA fragments encoding the pneumococcal neuraminidase, pJCP309 was digested with *Pst*I and fragments were randomly cloned into pBluescript SK. These were transformed into *E. coli* DH5α and screened for production of neuraminidase activity. One neuraminidase-positive subclone contained a pBluescript derivative (designated pJCP310) with a 5.2-kb *Pst*I insert. Interestingly, this insert did not hybridize (even at low stringency) with a digoxigenin-labeled probe specific for *nanA* (a 1.4-kb *Hin*dIII fragment from pMC4170 [10]). How-

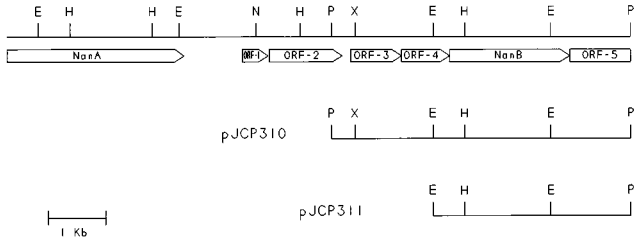

FIG. 1. Genetic map of a portion of the *S. pneumoniae* DNA insert of recombinant cosmid pJCP309. The horizontal lines represent *S. pneumoniae* DNA cloned in pJCP309 and subclones thereof cloned in pBluescript (pJCP310 and pJCP311). ORFs (determined by sequence analysis) are indicated below the map. Restriction sites are abbreviated as follows: E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; X, *Xho*I.

ever, pJCP309 DNA hybridized strongly to this probe, suggesting that the cosmid clone encoded two unrelated neuraminidases. Southern hybridization and restriction analysis with the *nanA* fragment and the insert of pJCP310 as probes were then used to map the positions of the two putative neuraminidase-encoding regions within the insert of pJCP309 (Fig. 1). Southern hybridization analysis also confirmed that the pattern of restriction sites in the cosmid was consistent with that for the homologous region of the serotype 6 pneumococcal chromosome (result not shown).

Sequence analysis. The complete nucleotide sequence of the insert of pJCP310 was then determined to characterize the second pneumococcal neuraminidase gene, which we have designated *nanB*. We also subcloned additional DNA fragments from pJCP309 to determine the sequence of the entire region between *nanA* and *nanB*. The 8,248-bp sequence deposited with GenBank extends from the *Hin*dIII site within the distal portion of the *nanA* coding sequence to the *Pst*I site downstream from *nanB*. The 5′ end of this sequence overlaps that reported by Camara et al. (10) for *nanA* (EMBL accession number X72967) by 619 bp. The region downstream from *nanA* contains five complete open reading frames (ORFs) (capable of encoding polypeptides of 16.8, 49.5, 33.6, 30.5, and 77.7 kDa), as well as the proximal portion of a sixth ORF, as shown in Fig. 1. All of the ORFs are preceded by ribosome-binding sites. The position of *nanB* within this region was deduced from the fact that *E. coli* DH5α carrying pJCP311, a subclone of pJCP310 containing a 3,368-bp pneumococcal DNA insert with only one complete ORF (Fig. 1), produced an active neuraminidase. This portion of the DNA sequence and the deduced amino acid sequence of NanB are shown in Fig. 2.

NanB is 698 amino acids long, including a putative 29-residue signal peptide. Comparison of the NanB sequence with that of NanA revealed negligible homology. However, comparison with other sequences deposited with GenBank indicated a limited degree of amino acid homology (30% identity over 169 residues) with the sialidase of *Clostridium septicum* (30). The region of greatest similarity (51% identity and 86% similarity over 51 residues) includes an R-I-P motif that is found in the active site of other bacterial neuraminidases (29). NanB also contains three copies of the aspartate box consensus motif S-X-D-X-G-X-T-W that is common to other neuraminidases (29).

Purification and characterization of NanB. NanB was purified from French pressure cell lysates of *E. coli* DH5α (pJCP311) by ion-exchange chromatography on DEAE-Sepharose CL-6B and then subjected to gel permeation chromatography on Sephacryl S200-HR and dye-ligand chromatography on RedA resin as described in Materials and Methods. SDS-PAGE analysis suggested that the final purified NanB had an apparent molecular mass of approximately 65 kDa (Fig. 3). This is somewhat lower than expected from the deduced amino acid sequence, which predicted a size of approximately 74.5 kDa for mature NanB after cleavage of the signal peptide. This discrepancy might be due to either additional N- or C-terminal posttranslational processing of NanB or anomalous behavior on SDS-PAGE. To examine this, SDS-PAGE-purified NanB was electroblotted onto an Immobilon P membrane and subjected to N-terminal amino acid sequence analysis. The 15 residues at the N terminus were N-E-L-N-Y-G-Q-L-S-I-S-P-I-F-Q, which agrees exactly with the predicted sequence after removal of the signal peptide.

Purified NanA and NanB, as well as extracts of *E. coli* carrying plasmid pJCP309 or pJCP311 (or the respective vectors alone) and an *S. pneumoniae* lysate, were then subjected to Western blot analysis with antiserum raised against NanA or NanB (Fig. 4). As expected, anti-NanA serum-reactive bands were seen only in the *E. coli*(pJCP309), purified NanA, and *S. pneumoniae* tracks. Anti-NanB serum labeled a species of approximately 65 kDa in the *E. coli*(pJCP309), *E. coli*(pJCP311), purified NanB, and *S. pneumoniae* tracks. There was no apparent labeling of any protein species in the tracks containing extracts of *E. coli* carrying the two cloning vectors employed. Moreover, there was no detectable immunological cross-reaction between NanA and NanB.

To compare their pH optima, we assayed appropriate dilutions of purified NanA and NanB by using the MUAN substrate in various buffers ranging from pH 2.5 to pH 9.5 (Fig. 5). The pH optimum for NanB was approximately 4.5, which was considerably lower than that for NanA (pH 6.5 to 7.0). Purified NanB had a specific activity of 37 U/mg of protein when assayed at its optimum pH, which is approximately 100-fold lower than that which we have previously reported for NanA (20).

DISCUSSION

We have demonstrated in this study that *nanB*, a gene encoding a second *S. pneumoniae* neuraminidase, is located on the pneumococcal chromosome approximately 4.5 kb downstream of *nanA. nanB* appears to be part of a large operon consisting of at least six ORFs. *nanA* is unlikely to be part of this operon, as there is a strong transcription termination sequence immediately downstream of this gene (10). All of the six ORFs downstream of *nanA* have strong ribosome-binding sites 6 to 8 bp 5′ to the initiation codon, but a consensus −10 and −35 promoter sequence was found only upstream (150 to 180 bp) of ORF1. Moreover, there were no obvious transcription termination sequences downstream of ORF1 to ORF4 or *nanB* (result not presented). The precise functions of ORF1 to ORF5 are not known, but comparison of their deduced amino acid sequences with those deposited with GenBank has provided some clues. ORF3 and ORF4 have a degree of homology (in both cases, approximately 30% identity over 280 amino acids) with MsmF and MsmG, respectively. These are membrane proteins which form part of a binding protein-dependent transport system in *S. mutans* that is responsible for multiple sugar metabolism (31). ORF2 contains a typical lipoprotein signal sequence and has a limited degree of homology (32% identity and 58% similarity over 50 amino acids) with MsmE, the putative sugar-binding protein of the *msm* locus. Thus, it is possible that *nanB* is part of an operon encoding cleavage of *N*-acetylneuraminic acid from host glycoproteins or glycolipids, as well as binding and transport of the sugar into the

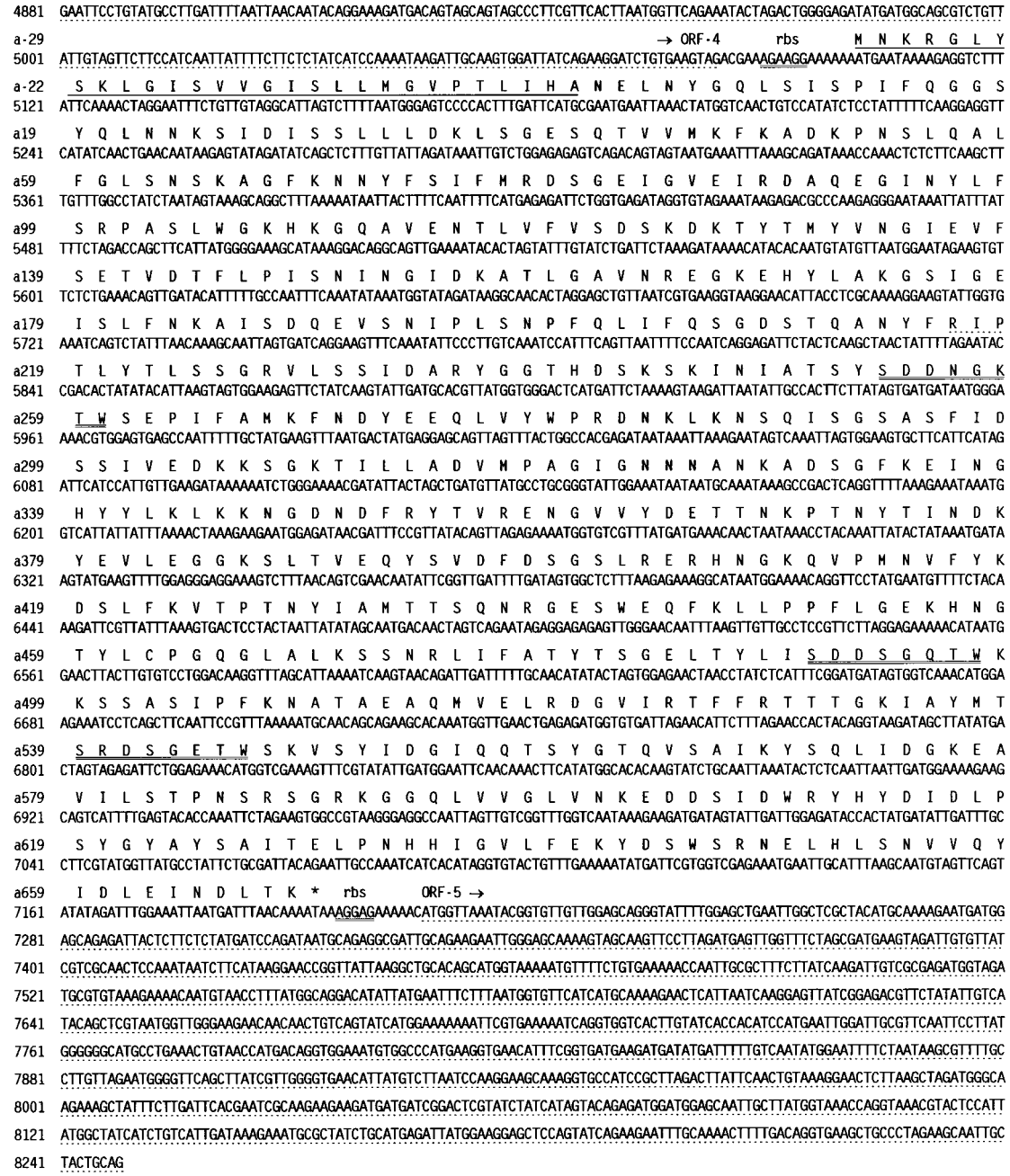

FIG. 2. Nucleotide sequence of the *S. pneumoniae* DNA insert in pJCP311 and deduced amino acid sequence of NanB. The DNA sequence is numbered in accordance with the sequence deposited with GenBank. Regions of the insert DNA encoding portions of ORF4 and ORF5 are dot underlined. Ribosome-binding sites (rbs) preceding *nanB* and ORF5 are double underlined. The deduced amino acid sequence of NanB is represented by single-letter code above the first nucleotide of each codon and is numbered from the first residue of the processed polypeptide chain, i.e., after removal of the signal peptide, which is underlined. Within the NanB sequence, the R-I-P motif, associated with the putative active site, is dot underlined and the three copies of the neuraminidase aspartate box are double underlined.

pneumococcus. The incomplete ORF5 has a high degree of homology (65% identity over 347 amino acids) with a hypothetical 41.9-kDa *E. coli* protein with an unknown function in the *leuX-fecE* intergenic region (GenBank accession number U14003).

The size of NanB (approximately 65 kDa), as judged by SDS-PAGE analysis of the purified enzyme, was smaller than that predicted by the sequence of *nanB*; after allowing for cleavage of the signal peptide, a size of 74.5 kDa was expected. This was not a consequence of incorrect assignment of the signal peptidase cleavage site, as this was confirmed by N-terminal amino acid sequence analysis of NanB purified from recombinant *E. coli*. The smaller-than-expected size was not an artifact of expression in *E. coli*, as Western blot analysis demonstrated that the apparent 65-kDa form was seen in both *S. pneumoniae* and recombinant *E. coli* lysates. Thus, the size discrepancy may be a consequence of anomalous behavior on SDS-PAGE, and/or posttranslational C-terminal processing.

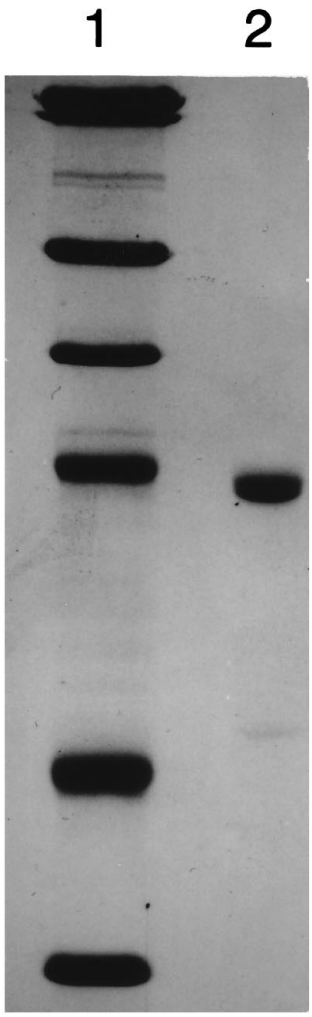

FIG. 3. SDS-PAGE analysis of purified NanB. Approximately 5 μg of NanB purified from *E. coli*(pJCP311) (lane 2) was electrophoresed on an SDS–12.5% PAGE gel as described in Materials and Methods and stained with Coomassie brilliant blue R-250. Lane 1 contained marker proteins with sizes (from top to bottom) of 200, 116, 97, 66, 45, and 31 kDa.

However, if C-terminal processing does take place, cleavage must occur at similar sites in *S. pneumoniae* and *E. coli*. Proteolytic processing is not unprecedented for pneumococcal neuraminidases, as we have previously reported that in *S. pneumoniae* lysates, NanA can be degraded from 107 kDa to a molecular mass as low as 86 kDa without obvious effect on enzymic activity (20). Also, Camara et al. (10) have reported that plasmid pMC2150, which lacks the 3′ portion of *nanA*, directs the production of active neuraminidase in *E. coli*. This truncated protein would be expected to lack 233 amino acids from the NanA C terminus. Our Western blot data indicate that proteolytic degradation of NanA also occurs in *E. coli*, as multiple immunoreactive fragments were detected in the lysate of *E. coli*(pJCP309).

The SDS-PAGE size estimate for NanB of 65 kDa is in close agreement with that reported for a neuraminidase purified from a serotype 6A strain of *S. pneumoniae* by Scanlon et al. (32). It is not possible, however, to determine whether this enzyme is NanB or a degradation product of NanA. Like NanB, its specific activity (after correction for differences in unit definition) is about 100-fold lower than that which we have reported for NanA (20). However, its pH optimum is intermediate between those of NanA and NanB. Moreover, its isoelectric point (pH 7.2) is clearly different from that predicted for NanA or the mature form of NanB (pHs 5.94 and 5.88, respectively). Unfortunately, amino acid composition or N-terminal sequence data, which would have resolved this question, were not presented.

The benefits to a pneumococcus of production of two distinct neuraminidases are unclear. Apart from their difference in size, the two enzymes have widely different pH optima, which implies that these enzymes may assist exploitation of distinct environmental niches. Although a clear difference in specific activity was observed with MUAN as the substrate, this may not hold for other potential substrates. NanA may not be more active than NanB in vivo if such differences in substrate preference exist. NanA and NanB are both exported proteins, with typical signal peptides, but unlike NanB, NanA contains a C-terminal cell surface anchorage domain, including the motif L-P-X-T-G-X, which is found in a number of gram-positive

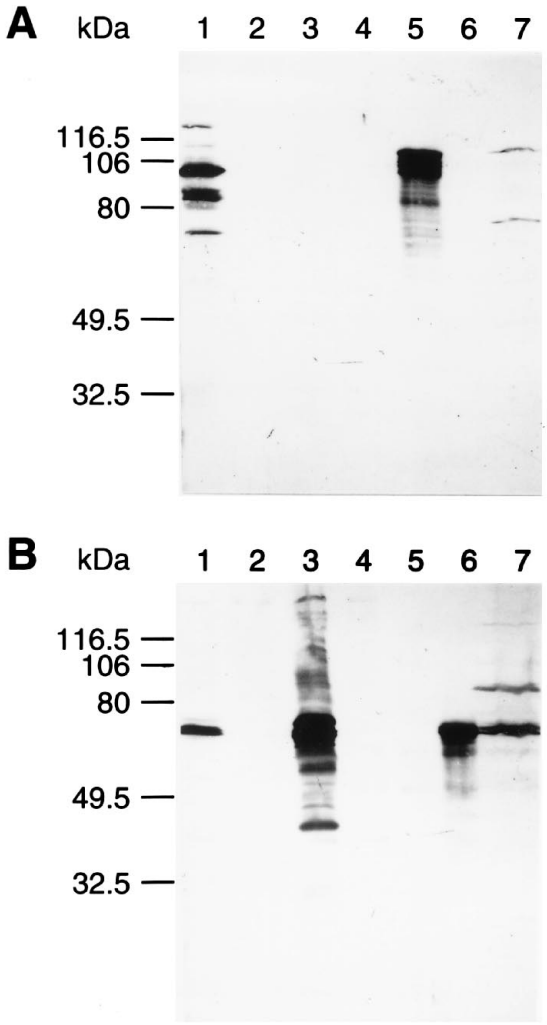

FIG. 4. Western blot analysis. Samples were subjected to SDS-PAGE, electroblotted, and probed with mouse anti-NanA (A) or mouse anti-NanB (B) serum as described in Materials and Methods. Lanes: 1, *E. coli* DH1(pJCP309) lysate; 2, *E. coli* DH1(pOU61cos) lysate; 3, *E. coli* DH5α(pJCP311) lysate; 4, *E. coli* DH5α(pBluescript) lysate; 5, purified NanA; 6, purified NanB; 7, *S. pneumoniae* serotype 6 lysate.

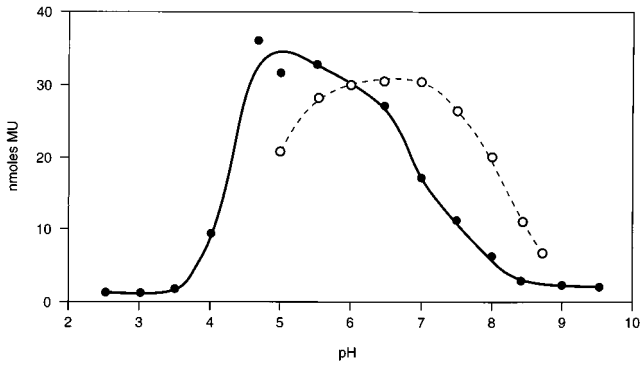

FIG. 5. pH optima of NanA and NanB. Neuraminidase activity was assayed by using MUAN as the substrate in buffers adjusted to the pHs indicated. Each assay contained 0.07 ng of purified NanA (○) or 7 ng of purified NanB (●). Activity is expressed as nanomoles of 4-methylumbelliferone (MU) released in 30 min at 37°C.

surface proteins (10, 33). Thus, a significant portion of NanA remains cell associated (20). Proteolytic cleavage without loss of enzymic activity may, in fact, be important for controlled release of surface-bound NanA. The possible involvement of neuraminidase in pneumococcal pathogenesis has been suggested by our previous finding that purified NanA is a partially protective immunogen in mice (21). However, it has not been possible to assess the contribution of neuraminidase to pneumococcal virulence by molecular genetic techniques, as NanA-deficient mutants have residual enzymic activity because of production of NanB (3a). The isolation and characterization of *nanB* in this study pave the way for a comprehensive assessment of the contribution of these enzymes to the pathogenesis of disease by construction of pneumococci carrying mutations in either *nanA*, *nanB*, or both.

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