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Identification of a Second *Arcanobacterium pyogenes* Neuraminidase and Involvement of Neuraminidase Activity in Host Cell Adhesion

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***Arcanobacterium pyogenes*, a common inhabitant of the upper respiratory and urogenital tracts of economically important animals, such as cattle and swine, is also an opportunistic pathogen associated with suppurative infections in these animals. *A. pyogenes* expresses neuraminidase activity encoded by the *nanH* gene, and previously, construction of a *nanH* mutant of *A. pyogenes* BBR1 indicated that a second neuraminidase is present in this strain. A 5,112-bp gene, *nanP*, was cloned and sequenced, and this gene conferred neuraminidase activity on an *Escherichia coli* host strain. The predicted 186.8-kDa NanP protein exhibited similarity to a number of bacterial neuraminidases and contained the RIP/RLP motif and five copies of the Asp box motif found in all bacterial neuraminidases. As expected, insertional inactivation of the *nanP* gene in *A. pyogenes* BBR1 resulted in a mutant with reduced neuraminidase activity. However, insertional inactivation of the *nanP* gene in the *nanH* mutant strain resulted in a complete lack of neuraminidase activity. Like NanH, NanP was localized to the *A. pyogenes* cell wall. However, unlike the *nanH* gene, which was present in 100% of the strains examined, *nanP* was present in only 64.2% of the isolates ($n = 53$). *A. pyogenes* adheres to HeLa cells, and a *nanP* mutant displayed a wild-type adhesion phenotype with these cells. In contrast, the ability of a *nanH nanP* double mutant to bind to HeLa cells was reduced by 53%. The wild-type adhesion phenotype was restored by providing *nanP* in *trans*. These data indicate that the neuraminidases of *A. pyogenes* play a role in adhesion of this organism to host epithelial cells.**

Adhesion to epithelial cells is necessary for bacteria to colonize host mucosal surfaces. This adhesion is the result of interaction of a number of surface-exposed or secreted bacterial proteins with host cells and molecules. Neuraminidases can be important factors in promoting adhesion to host epithelial cells in *Streptococcus pneumoniae* (10, 37) and oral *Actinomyces* spp. (4, 7). Neuraminidase treatment of whole-organ perfusion cultures of chinchilla tracheae increased *S. pneumoniae* adhesion and reversed inhibition of glycoconjugate analogs of known *S. pneumoniae* receptors (37). In *Actinomyces naeslundii*, adhesion to human epithelial or skin fibroblast cells mediated by type 2 fimbriae could be increased by pretreatment of the cells with neuraminidase (4). Similarly, pretreatment of human buccal epithelial cells with neuraminidase significantly increased the adhesion of *Actinomyces israelii*, *A. naeslundii*, and *Actinomyces viscosus* (7). In addition, the action of neuraminidase can decrease mucus viscosity (11), possibly enhancing bacterial colonization of the underlying tissues. Furthermore, the susceptibility of mucosal immunoglobulin A to bacterial proteases is increased when sialic acid moieties are removed from this molecule (9, 28).

Arcanobacterium pyogenes is a common inhabitant of the upper respiratory, urogenital (6, 35), and gastrointestinal 19; B. H. Jost, K. W. Post, and S. J. Billington, unpublished data) tracts of many domestic animal species. However, this organism can cause disease, usually following a physical or microbial insult to the host. *A. pyogenes* causes a variety of suppurative infections of the skin, joints, and visceral organs in economically important animals and birds, such as mastitis in dairy cows (14) and goats (1), liver abscesses in feedlot cattle (17, 18), pneumonia in pigs (12), and osteomyelitis in turkeys (5).

A. pyogenes expresses neuraminidase activity, and this activity is in part due to NanH, a cell wall-bound neuraminidase found in all isolates (16). Construction of a *nanH* insertion deletion mutant in *A. pyogenes* resulted in a strain with reduced neuraminidase activity, but neuraminidase activity was still present, suggesting that a second enzyme was present (16). The *nanH* mutant was not defective for adhesion to epithelial cells (16), but the role of NanH in adhesion of *A. pyogenes* to host cells may have been masked by the presence of the second neuraminidase.

In this paper we describe cloning and characterization of NanP, a second neuraminidase expressed by *A. pyogenes*. In addition, we constructed a *nanH nanP* double mutant and determined that neuraminidase activity plays a role in the adhesion of *A. pyogenes* to host epithelial cells.

MATERIALS AND METHODS

Bacteria and growth conditions. *A. pyogenes* strain BBR1 was isolated from a bovine abscess. *A. pyogenes* strain NANH-1, in which the entire *nanH* gene was deleted and replaced with an erythromycin resistance cassette, was described previously (16). Other *A. pyogenes* strains used in this study were obtained from veterinary diagnostic laboratories or personal collections. *A. pyogenes* strains were grown on brain heart infusion (Difco) agar plates supplemented with 5% bovine blood at 37°C in the presence of 5% CO_2 or in brain heart infusion broth supplemented with 5% bovine calf serum (Omega Scientific Inc.) at 37°C with shaking. *Escherichia coli* DH5αMCR strains (Gibco-BRL) were grown at 37°C on Luria-Bertani (Difco) agar or in Luria-Bertani broth with shaking. The following antibiotics (Sigma) were added as appropriate: for *A. pyogenes* strains, chloramphenicol (5 μg/ml), erythromycin (15 μg/ml), and kanamycin (30 μg/ml); and for *E. coli* strains, chloramphenicol (30 μg/ml) and kanamycin (50 μg/ml).

Preparation of CSF, CWE, and protoplasts. Culture supernatant fluid (CSF) was prepared from liquid cultures of *A. pyogenes* grown overnight to optical

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densities at 600 nm of approximately 3.0 to 4.0. Cells were removed by centrifugation at 5,000 × *g*, and the CSF was filtered through a 0.22-μm-pore-size filter. *A. pyogenes* cell wall extract (CWE) and protoplasts were prepared as previously described for *S. pneumoniae* (20). Protoplasts were resuspended in distilled water and were lysed by several cycles of freezing and thawing.

DNA techniques. Preparation of plasmid DNA and electroporation-mediated transformation of *A. pyogenes* strains were performed as previously described (15). Genomic DNA from *A. pyogenes* was isolated by the method of Pospiech and Neumann (24). The methods used for growth and purification of bacteriophage were essentially the methods described by Ausubel et al. (3). DNA was prepared from bacteriophage as previously described (32) and was further purified by using a Wizard DNA Clean-Up system (Promega). *E. coli* plasmid DNA extraction, transformation, DNA restriction, ligation, agarose gel electrophoresis, and Southern transfer of DNA to nitrocellulose membranes were performed essentially as described previously (3). Preparation of DNA probes, DNA hybridization, and probe detection were performed by using a DIG DNA labeling and detection kit (Roche Molecular Biochemicals) as recommended by the manufacturer. PCR DNA amplification was performed by using *Taq* DNA polymerase (Promega) with the supplied reaction buffer for 35 cycles consisting of 1 min at 94°C, 1 min at 55°C, and 1 min/kb at 72°C, followed by a final extension step of 72°C for 5 min.

Nucleotide sequence determination. The sequence of *nanP* was determined with plasmid pJGS360 by automated DNA sequencing. Sequencing was performed for both strands; all restriction sites were crossed, and KS or T7 sequencing primers or oligonucleotide primers (Sigma-Genosys) designed to sequence *nanP* were used. Sequencing reactions were performed at the DNA Sequencing Facility of The University of Arizona with a 377 DNA sequencer (Applied Biosystems Inc.).

Computer sequence analysis. Nucleotide sequence data were compiled by using the Sequencher program (GeneCodes). Database searches were performed by using the BlastX and BlastP algorithms (2). Sequence analysis was performed by using the suite of programs available through the Genetics Computer Group, Inc. (University of Wisconsin). Signal sequence prediction was performed by using SignalP (22). Multiple sequence alignments were performed by using CLUSTAL W (34).

Detection of neuraminidase activity. Neuraminidase activity was assayed by using the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN) (Sigma), essentially as described by Winter et al. (38), in 100 mM citrate phosphate buffer (pH 6.0) at 37°C for 1 h.

A λGEM-12 library of *A. pyogenes* BBR1 genomic DNA was plated, as described above, so that approximately 300 plaques were present on an 8.5-cm-diameter petri dish. An overlay consisting of 2.5 ml of top agar (3), 75 μl of 1.0 M sodium acetate (pH 4.5), and 500 μl of 0.35% MUAN was poured over the plate and allowed to set. The plate was incubated at 4°C for 30 min prior to excitation with a UV source (254 nm), and the presence of neuraminidase activity was indicated by cyan fluorescence.

Screening of bacterial strains for neuraminidase activity was performed by using a filter paper assay. No. 2 filter paper (Whatman) was wetted with 100 μM MUAN in 0.1 M sodium acetate (pH 4.5). Colonies were patched onto the filter paper and incubated at 37°C for 15 min prior to excitation with UV light, as described above.

Epithelial cell adhesion assay. Human cervical epithelial cells (HeLa cells) were cultured in Iscove's modified Dulbecco's medium (Life Technologies) supplemented with 10% fetal bovine serum (Omega Scientific Inc.) and with 100 μg of gentamicin (Sigma) per ml in a humidified, 5% CO_2 atmosphere at 37°C. For adhesion assays, HeLa cells in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum without gentamicin were seeded into 24-well plates at a concentration of 2×10^5 cells per well in 1-ml volumes. The cells were incubated at 37°C in the presence of 5% CO_2 for 18 h prior to addition of 2×10^6 bacteria (freshly grown to an optical density at 600 nm of 1.0). Bacterial adhesion was assessed after 1 h of incubation at 37°C in the presence of 5% CO_2. Cell monolayers were washed three times with 0.01 M phosphate-buffered saline (pH 7.2) to remove nonadherent bacteria. Bacteria were recovered by treatment of the cell monolayers with 1 ml of 0.1% Triton X-100 (Sigma) for 10 min at 0°C, and viable bacteria were enumerated by dilution plating. All experiments were performed in triplicate on three separate occasions.

Statistical analysis. A one-way analysis of variance was performed with the data from the epithelial cell adhesion assays by using the Minitab statistical software (Minitab Inc.).

Nucleotide sequence accession number. The *nanP* sequence has been deposited in the GenBank database under accession number AY045771.

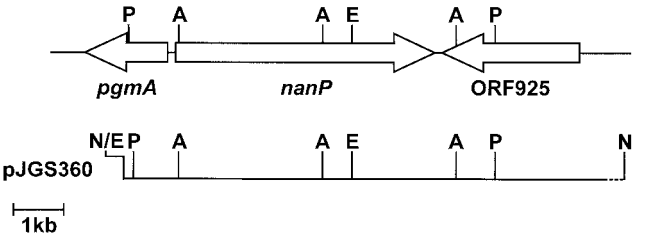

FIG. 1. Map of the *nanP* gene region. A map of plasmid pJGS360 is shown below the gene region map. *Asp*700I (A), *Eco*RI (E), *Not*I (N), and *Pst*I (P) sites used to clone portions of the *nanP* gene are shown.

RESULTS

Cloning and determination of the nucleotide sequence of *nanP*. An *A. pyogenes* BBR1 *nanH* insertion deletion mutant exhibited neuraminidase activity (16), suggesting that a second neuraminidase gene was present in this strain. Plaques from a λGEM-12 library of *A. pyogenes* BBR1 genomic DNA (16) were overlaid with MUAN-containing top agar and were visualized under UV light. Several strongly fluorescent plaques were selected and used for PCR analysis. One of these bacteriophages, λJGS37, was not amplified with *nanH*-specific primers (data not shown) and contained an approximately 15-kb partial *Sau*3AI fragment. DNA purified from λJGS37 was digested with *Not*I and cloned into similarly digested pBC KS (Stratagene). Plasmid DNA from one of the recombinants contained an approximately 10-kb *Not*I fragment (pJGS360) encompassing the entire *nanP* gene region (Fig. 1). pJGS360 conferred neuraminidase activity on the *E. coli* host, as determined by the MUAN filter paper assay.

The DNA sequence of the *nanP* gene region was deduced from pJGS360. There are several in-frame ATG codons at the 5′ end of the *nanP* gene. The first and second ATG codons appear to be equal candidates, as they both have consensus ribosome binding sites. If it was assumed that translation occurred from the first ATG, the 5,211-bp *nanP* gene encoded a protein with a predicted molecular mass of 186.8 kDa. A gram-positive signal sequence, with a cleavage site between Ala-50 and Glu-51, was predicted by SignalP (22). A putative *rho*-independent terminator ($\Delta G = -18.4$ kcal/mol) was identified 25 bases downstream of the *nanP* stop codon. No *E. coli* σ^{70}-like promoter sequences were apparent upstream of the *nanP* gene.

Upstream of *nanP* was an open reading frame (ORF), *pgmA*, whose protein product was similar to phosphoglucomutase from *Streptomyces coelicolor* (26). Downstream sequences encoded an ORF designated ORF925, whose protein product was similar to a putative integral membrane protein from *S. coelicolor* (26). *pgmA* and ORF925 were transcribed in the direction opposite the direction of *nanP* transcription (Fig. 1), suggesting that *nanP* is monocistronic.

Analysis of the primary structure of NanP. Cleavage at the predicted signal peptide sequence of NanP should result in a mature protein with a predicted molecular mass of 181.6 kDa and a pI of 5.4. The NanP protein contained the conserved catalytic RIP/RLP motif, as well as five copies of the Asp box motif (Ser-x-Asp-x-Gly-x-Thr-Trp) associated with bacterial

neuraminidases (29) (Fig. 2). NanP was most similar to NedA from *Micromonospora viridifaciens* (45.3% identity, 61.6% similarity), NanH from *A. viscosus* (43.1% identity, 59.4% similarity), and NanH from *A. pyogenes* (38.8% identity, 53.8% similarity) (Fig. 2).

At the C terminus of the NanP protein was a sequence similar to the cell wall sorting signals found in surface-expressed proteins of gram-positive bacteria (33). The terminal 32 amino acids of NanP (**LAWTG**AAVVGLAVMSLVFL LAGFVLTV*RRRK*A) consisted of an LPxTG cleavage motif (boldface), a hydrophobic domain (underlined), and a positively charged stop transfer sequence (italics). However, in the *A. pyogenes* NanP protein, the cleavage motif was LAWTG instead of LPxTG. In addition, a Pro-rich repetitive region, which is thought to facilitate spanning of the cell wall peptidoglycan (33), was found directly upstream of the LAWTG cleavage motif.

Determination of the prevalence of the *nanP* gene by DNA dot blotting. In order to determine whether *nanP* was present in all *A. pyogenes* strains, genomic DNAs were prepared from 53 *A. pyogenes* strains and were subjected to hybridization under high-stringency conditions with a *nanP*-specific probe that spanned bases 143 to 1385 of the *nanP* ORF, encompassing the active site region of the molecule. As a control, pJGS306, encoding the entire *nanH* ORF except the signal sequence, was tested (16). The *nanP* probe did not hybridize to *nanH* sequences (data not shown), indicating that the probe was specific for *nanP*. Genomic DNA from 34 of the 53 strains hybridized strongly to the probe (data not shown), indicating that the *nanP* gene is present in 64.2% of the *A. pyogenes* strains tested. Furthermore, *nanP* was found preferentially in *A. pyogenes* isolates of bovine origin. Of 31 bovine *A. pyogenes* isolates, 24 (77.4%) were *nanP* positive, compared with only 5 of 17 porcine isolates (29.4%). In addition, 7 of the 53 strains hybridized weakly, but consistently, to the *nanP* probe (data not shown), suggesting that a different but related neuraminidase may be present in these *A. pyogenes* strains.

Construction and characterization of *nanP* mutants. To construct *nanP* mutants, we used an allelic exchange plasmid in which the *nanP* gene was inactivated by deletion of the active site region of the *nanP* ORF and insertion of a kanamycin resistance gene (Fig. 3). The 4.5-kb *Eco*RI fragment of pJGS360 (Fig. 1) was cloned into pHSS21 (21) to form the recombinant plasmid pJGS401. A 1.4-kb *Hin*dIII fragment containing the kanamycin resistance gene from pKRP11 (27) was treated with T4 DNA polymerase (Promega). The internal 2.9-kb *Asp*700I fragment (containing the active site region) of pJGS401 was replaced with a kanamycin resistance gene fragment, resulting in pJGS403. The *Eco*RI insert of pJGS403 was then cloned into similarly digested pBC KS to construct pJGS407 (Fig. 3). As pJGS407 was based on a ColE1 replicon, it acted as a suicide plasmid in *A. pyogenes* (15). pJGS407 plasmid DNA was introduced into *A. pyogenes* BBR1 or NANH-1 cells by electroporation, and recombinants were selected on brain heart infusion blood agar containing kanamycin. Km^r Cm^s colonies were chosen and used for further analysis.

Southern blotting of *A. pyogenes* BBR1 genomic DNA digested with *Pst*I revealed a hybridizing band at 7.2 kb in BBR1 when the preparation was probed with a *nanP*-specific probe (spanning bases 143 to 1385 of the *nanP* ORF). No hybridizing bands were observed for the *nanP* mutant (NANP-1) or the *nanH nanP* double mutant (NANHP-1) when they were probed similarly, indicating that the *nanP* active site region was not present in these strains. A 5.7-kb band was apparent in *Pst*I-digested NANP-1 or NANHP-1 DNA but not BBR1 genomic DNA when a kanamycin resistance gene-specific probe was used. None of the strains hybridized with a pBC KS-specific (vector) probe (data not shown). These data confirmed that deletion or inactivation of the *nanP* gene occurred in NANP-1 and NANHP-1 by double-crossover events.

Complementation of the NANHP-1 double mutant. In order to construct a complementing plasmid, the 7.2-kb *Pst*I fragment of pJGS360, containing *nanP*, was cloned into similarly digested pJGS180 to construct pJGS398. pJGS180 is a derivative of pEP2 (25) carrying a chloramphenicol resistance gene, and this plasmid can replicate in *A. pyogenes* (S. J. Billington, unpublished data). pJGS398 was introduced into NANHP-1 by electroporation with selection on chloramphenicol. The neuraminidase activities of NANP-1, NANHP-1, NANHP-1(pJGS180), and NANHP-1(pJGS398) were compared to the neuraminidase activity of wild-type strain BBR1 by using a MUAN-filter paper assay. BBR1 and the NANP-1 mutant expressed neuraminidase activity, but neither NANHP-1 nor NANHP-1(pJGS180) had detectable neuraminidase activity. NANHP-1(pJGS398) expressed neuraminidase activity, indicating that neuraminidase activity could be restored by providing *nanP* in *trans* on a replicating plasmid (data not shown).

Localization of NanP. Whole cells, CSF, and CWE were prepared from wild-type strain BBR1 and the NANP-1 and NANHP-1 mutants, and these preparations were tested for the presence of neuraminidase activity with the fluorometric assay by using MUAN as a substrate. For BBR1 and NANP-1 the majority of the neuraminidase activity was detected in whole cells and CWE. While BBR1 cells had only slightly higher neuraminidase activity than NANP-1 cells, BBR1 CWE had significantly higher neuraminidase activity than the CWE from the NANP-1 mutant (Fig. 4). Some neuraminidase activity was detected in the CSF and in lysed protoplasts of either BBR1 or NANP-1 (Fig. 4), and this activity may have resulted from fragments of cell wall material that were present in the CSF or were still associated with the protoplasts. These data indicate that the majority of NanP-specific neuraminidase activity was associated with the cell wall. No significant neuraminidase activity was detected in any of the NANHP-1 fractions (Fig. 4).

Adhesion of *A. pyogenes* neuraminidase mutants to HeLa cells. The abilities of BBR1, NANH-1, NANP-1, NANHP-1, and the complemented NANHP-1 mutant to adhere to HeLa cells were determined. In the initial experiments centrifugation following addition of bacteria to HeLa cells was used to increase bacterium-host cell interactions (16). However, this step could potentially result in bacterium-host cell adhesion and mask subtle differences between wild-type and neuraminidase-deficient *A. pyogenes* strains, so subsequent experiments were performed without centrifugation. The NANHP-1 double mutant exhibited impaired adhesion compared to wild-type strain BBR1; the average levels of adhesion were 5.9 and 12.6%, respectively (Fig. 5). The lower level of adhesion was reproducible and statistically significant ($P < 0.001$). The adhesion phenotypes of the NANH-1 and NANP-1 mutants were the

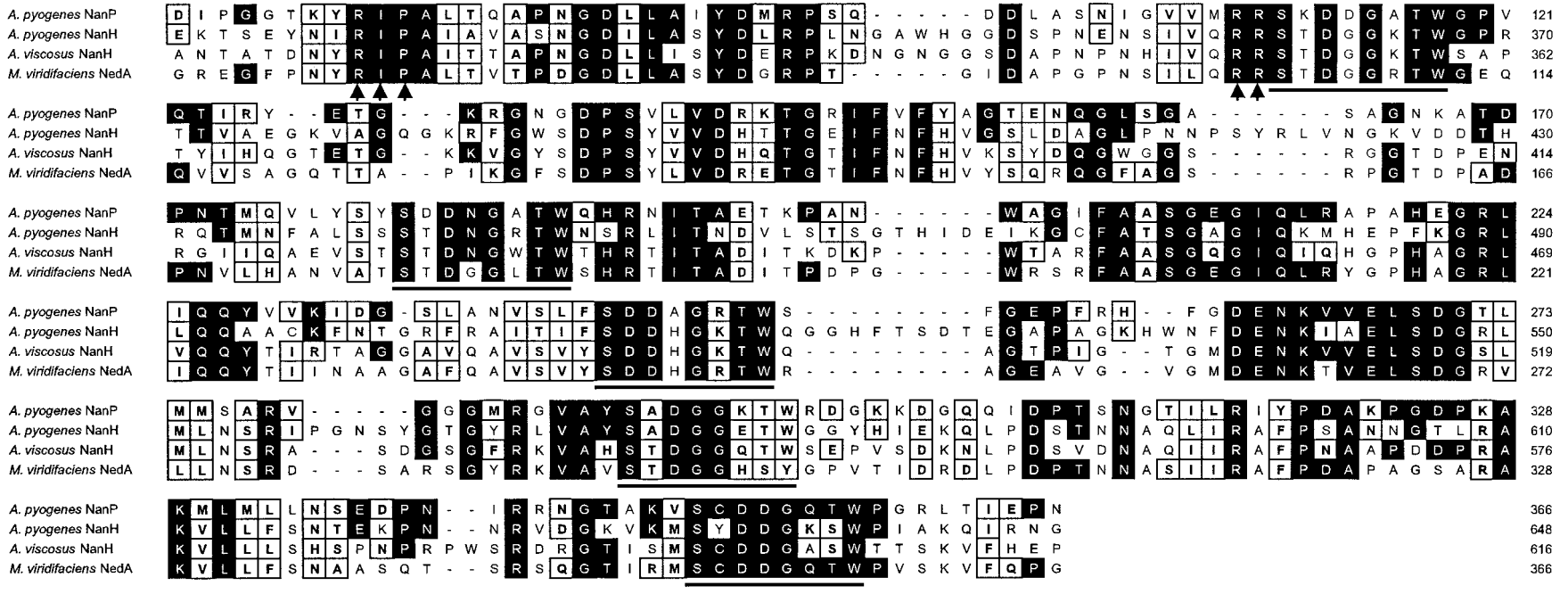

FIG. 2. Amino acid alignment for the active site regions of *A. pyogenes* NanP and NanH (16), *A. viscosus* NanH (39), and *M. viridifaciens* NedA (31). Amino acids identical to amino acids in the NanP sequence are indicated by black boxes. Conservative substitutions are indicated by open boxes. The RIP/RLP motif and the two additional catalytic Arg residues are indicated by arrows. The Ser-x-Asp-x-Gly-x-Thr-Trp (Asp box) motifs are underlined. Amino acid numbers for each protein are indicated on the right.

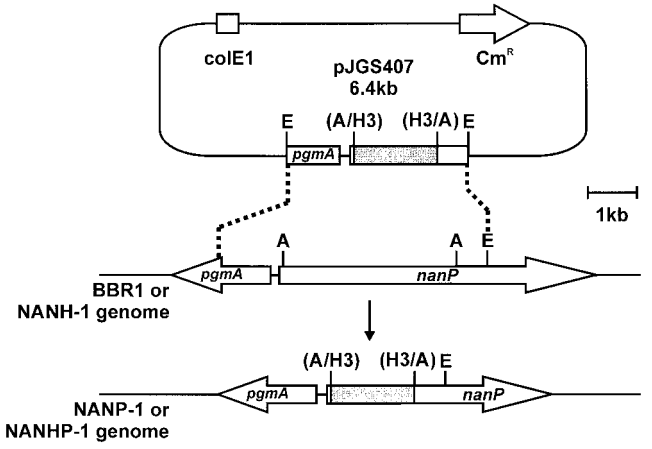

FIG. 3. Scheme for insertional inactivation of the *A. pyogenes nanP* gene. An *Asp*700I fragment containing the active site region of *nanP* was replaced with a Km^r cassette to construct pJGS407. This plasmid was introduced into *A. pyogenes* strains BBR1 and NANH-1 by electroporation. Reciprocal recombination, indicated by the dashed lines, resulted in replacement of the active site region of the *nanP* gene in the BBR1 and NANH-1 chromosomes with the Km^r cassette to construct NANP-1 and NANHP-1, respectively. A, *Asp*700I; E, *Eco*RI; H3, *Hin*dIII. Only the insert portion of pJGS407 is to scale. The *Eco*RI site in pJGS407 was derived from the vector portion of pJGS360.

same as the adhesion phenotype of BBR1, and the average levels of adhesion were 12.0 and 12.7%, respectively (Fig. 5). Complementation of the NANHP-1 mutant with pJGS398, carrying *nanP*, resulted in a wild-type adhesion phenotype (average level of adhesion, 12.5%). If centrifugation was used, no significant difference in adhesion between BBR1 and NANHP-1 was observed (data not shown). These findings suggest that a complete absence of neuraminidase activity does impair the ability of *A. pyogenes* to adhere to HeLa cells.

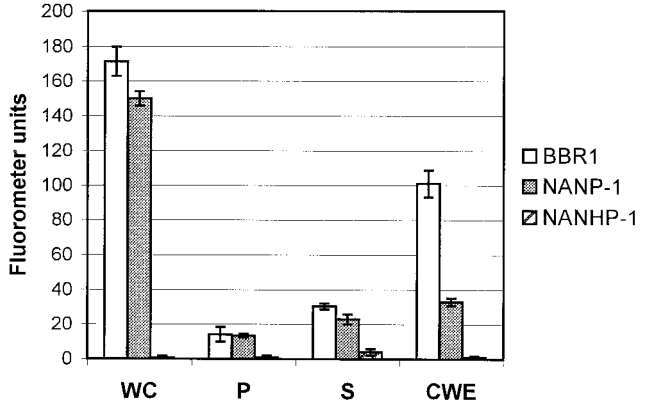

FIG. 4. Cell localization of neuraminidase activity. A total of 4×10^7 whole cells (WC), 4×10^7 lysed protoplasts (P), 20 μl of culture supernatant (S), and 200 μg of purified CWE from wild-type BBR1 (open bars), the NANP-1 mutant (gray bars), and the NANHP-1 mutant (cross-hatched bars) were assayed for neuraminidase activity by using the fluorometric assay with MUAN as a substrate. The error bars indicate 1 standard deviation of the mean calculated by using the averages from at least three independent experiments.

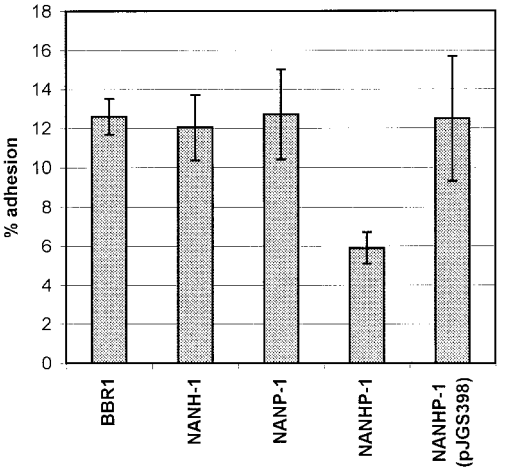

FIG. 5. Adhesion of *A. pyogenes* strains to HeLa cells. *A. pyogenes* strains were added to cell monolayers and allowed to adhere for 1 h at 37°C prior to washing and recovery of cell-associated bacteria. Adhesion is expressed as a percentage of the number of bacteria originally added to the cells. The error bars indicate 1 standard deviation of the mean calculated by using the averages from at least three independent experiments performed in triplicate.

DISCUSSION

We previously characterized NanH, a neuraminidase from *A. pyogenes* BBR1, and demonstrated that a *nanH* mutant exhibited neuraminidase activity and showed no reduction in adhesion to HeLa cells. In this paper we describe cloning and sequencing of NanP, a second neuraminidase from *A. pyogenes* BBR1. Like the NanH neuraminidase, NanP is present in the cell wall. However, unlike the *nanH* gene, *nanP* is carried by only 64.2% of the *A. pyogenes* isolates examined.

Sequence analysis of the *nanP* gene region indicated that *nanP* was monocistronic and flanked by *pgmA,* a phosphoglucomutase, and ORF925, which encodes a putative integral membrane protein, which were identified by their similarity to *S. coelicolor* genes (26). NanP contained sequences consistent with its activity as a neuraminidase, including the RIP/RLP and Asp box motifs (29). Like NanH, NanP is most closely related to NedA of *M. viridifaciens* (31) and NanH of *A. viscosus* (39). Surprisingly, the *A. pyogenes* NanH and NanP proteins exhibit less similarity to each other than either of them exhibits to the neuraminidases of other species. In addition, the *nanH* and *nanP* genes do not align, nor do they hybridize under high-stringency conditions, suggesting that it is unlikely that these genes arose through gene duplication in *A. pyogenes*.

The finding that NanP is localized to the *A. pyogenes* cell wall is consistent with the presence of an N-terminal signal peptide and C-terminal cell sorting signals, including an LPxTG-like cell anchor (33). NanP, like NanH, has a variant cell wall anchor motif, LAWTG. It is now clear that many cell wall-sorted proteins from other gram-positive bacteria also have divergent LPxTG motifs (23).

As expected, a *nanP* mutant, NANP-1, exhibited significant neuraminidase activity due to the presence of *nanH*. However, a *nanH nanP* double mutant, NANHP-1, completely lacked neuraminidase expression, a defect which could be reversed by providing *nanP* in *trans* on a replicating plasmid, pJGS398.

Like NanH, NanP was localized to the cell wall, as NANP-1 CWE had significantly reduced neuraminidase activity (Fig. 4). The neuraminidase activity of whole cells of NANP-1 was only slightly lower than the neuraminidase activity of wild-type BBR1 (Fig. 4). This was expected, as deletion of the *nanH* gene resulted in an approximately 80% reduction in the total amount of neuraminidase activity compared with the total amount of neuraminidase activity in BBR1 (16).

In contrast to *nanH*, which was present in all *A. pyogenes* isolates, *nanP* was found in only 64.2% of the strains examined. Interestingly, *nanP* appeared to be preferentially associated with *A. pyogenes* isolates of bovine origin; 77.4% of bovine strains carried *nanP*, compared to 29.4% of porcine isolates. In addition, some strains hybridized weakly, but consistently, to the *nanP* probe (data not shown), suggesting that a different but related neuraminidase is present in these *A. pyogenes* strains. Whether the presence of different neuraminidases suggests different host specificities requires further investigation. However, while there is some diversity in the number and types of neuraminidase genes present in *A. pyogenes*, all strains do express neuraminidase activity due to the presence of at least *nanH*.

Attachment to mammalian cells via specific recognition structures is the first step in bacterial colonization of a host. Adherence of *A. naeslundii* to epithelial cells (4) and polymorphonuclear leukocytes (30) was enhanced by pretreatment of the cells with neuraminidase. Similarly, neuraminidase treatment of tracheal organ cultures increased the adherence of *S. pneumoniae* (37), and *S. pneumoniae* mutants deficient in neuraminidase activity had reduced abilities to colonize and persist in the nasopharynx (36). A *nanH* mutant of *A. pyogenes* was not deficient in adhesion to HeLa cells (16), presumably due to the presence of the NanP neuraminidase. Similarly, the *nanP* mutant NANP-1 had a wild-type adhesion phenotype. In contrast, the ability of NANHP-1, which had no detectable neuraminidase activity, to adhere to HeLa cells was reduced 53%, indicating that neuraminidase activity was required for complete adhesion. The level of adhesion of NANHP-1 could be increased to the wild-type level by introducing *nanP* on a plasmid. No effect of *nanP* copy number was observed. In fact, the NANH-1 or NANP-1 mutant or complemented NANHP-1 adhered as well as BBR1, which suggests that only a defined amount of neuraminidase is required for complete adhesion.

While our results demonstrate that the neuraminidases play a role in adhesion to host cells, adhesion of *A. pyogenes* to host epithelial cells is probably a complex process, and several adhesins or ligands may contribute to it. The finding that adhesion of a neuraminidase-deficient mutant was reduced but was not absent suggests that there are adhesins which act in a neuraminidase-independent manner. Some of these adhesins could be cell wall proteins, including fibronectin- or collagen-binding proteins, such as the microbial surface components recognizing adhesive matrix molecules present in *Streptococcus pyogenes* and *Staphylococcus aureus* (8, 13). Furthermore, the data do not preclude the possibility that the neuraminidase activity that promotes adhesion of *A. pyogenes* to host cells plays a more prominent role in vivo.

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