Cloning, Expression, and Characterization of a Neuraminidase Gene from *Arcanobacterium pyogenes*

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Received 21 February 2001/Returned for modification 13 April 2001/Accepted 23 April 2001

*Arcanobacterium pyogenes* is an opportunistic pathogen, associated with suppurative infections in domestic animals. In addition to pyolysin, a pore-forming, cholesterol-binding toxin, *A. pyogenes* expresses a number of putative virulence factors, including several proteases and neuraminidase activity. A 3,009-bp gene, *nanH*, was cloned and sequenced and conferred neuraminidase activity on an *Escherichia coli* host strain. The predicted 107-kDa NanH protein displayed 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. Recombinant His-tagged NanH was found to have pH and temperature optima of 5.5 to 6.0 and 55°C, respectively. Insertional deletion of the *nanH* gene resulted in the reduction, but not absence, of neuraminidase activity, indicating the presence of a second neuraminidase gene in *A. pyogenes*. NanH was localized to the *A. pyogenes* cell wall. *A. pyogenes* adhered to HeLa, CHO, and MDBK cells in a washing-resistant manner. However, the *nanH* mutant was not defective for adherence to epithelial cells. The role of NanH in host epithelial cell adherence may be masked by the presence of a second neuraminidase in *A. pyogenes*.

*Arcanobacterium pyogenes* is a common inhabitant of the upper respiratory, urogenital (12, 54), and gastrointestinal tracts (35; B. H. Jost, K. W. Post, and S. J. Billington, unpublished data) of many domestic animal species. However, a physical or microbial insult to the host can lead to a variety of supplicative *A. pyogenes* infections, such as mastitis in dairy cows (27) and goats (2), liver abscesses in feedlot cattle (31, 34), and pneumonia in pigs (26) and various species of wildlife (17, 42, 57). *A. pyogenes* can also infect avian species (10) and humans (5, 16, 19), although infections in humans are rare.

*A. pyogenes* elaborates a number of extracellular proteins, including the hemolytic exotoxin pyolysin (PLO) (8), several proteases (48, 51), a DNase (30), and at least one neuraminidase (47). While all these proteins are putative virulence factors, only for PLO is there definitive evidence of involvement in the pathogenesis of infections by *A. pyogenes* (29).

Recently, there has been interest in the neuraminidases of bacterial pathogens and the potential role they play in pathogenesis. Neuraminidase (N-acetylneuraminyl hydrolase; EC 3.2.1.18) removes sialic acid from glycolipids, glycoproteins, and poly- and oligosaccharides. Bacterial neuraminidases have only 20 to 30% amino acid sequence identity but contain two conserved motifs, the RIP/RLP motif (Arg-Ile/Leu-Pro) and the Asp box motif (Ser-Asp-X-Gly-X-Thr-Trp), which occurs four or five times in the enzyme (14, 21, 43).

Neuraminidases are virulence factors, especially in bacteria that inhabit mucosal surfaces (20, 22, 52, 55), and they may play several roles in virulence. This enzyme can make sialic acid available as a carbon source to promote growth in a nutrient-limited environment (11, 23). The action of neuraminidase can decrease mucus viscosity (24), possibly enhancing colonization of the underlying tissues. Desialylation by neuraminidase can increase the susceptibility of mucosal IgA to bacterial proteases (18, 41). Neuraminidase can enhance bacterial adhesion and colonization (9, 13, 22, 56) and susceptibility of the host to the action of toxins (20), by exposing cryptic host cell receptor molecules.

This paper describes the cloning and characterization of a neuraminidase expressed by *A. pyogenes*. In addition, we show that *A. pyogenes* can adhere to epithelial cells in a washing-resistant manner, and we have investigated whether neuraminidase plays a role in this adhesion.

**MATERIALS AND METHODS**

**Bacteria and growth conditions.** *A. pyogenes* strain BBR1 was isolated from a bovine abscess. Other *A. pyogenes* strains used in this study were from veterinary diagnostic laboratories or personal collections. *A. pyogenes* strains were grown on brain heart infusion (BHI; Difco) agar plates, supplemented with 5% bovine blood, at 37°C and 5% CO2 or in BHI broth supplemented with 5% bovine calf serum at 37°C with shaking. *Escherichia coli* DH5αMCR strains (Gibco-BRL) were grown at 37°C on Luria-Bertani (LB; Difco) agar or in LB broth with shaking. Antibiotics (Sigma) were added as appropriate; for *A. pyogenes* strains, erythromycin (EM) at 15 μg/ml and kanamycin (KM) at 30 μg/ml; for *E. coli* strains, ampicillin at 100 μg/ml, chloramphenicol at 30 μg/ml, EM at 200 μg/ml, and KM at 50 μg/ml.

**Preparation of CSF, CWE, and protoplasts.** Culture supernatant fluid (CSF) was prepared from liquid cultures of *A. pyogenes* grown overnight to an optical density at 600 nm (OD<sub>600</sub>) 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 *Streptococcus pneumoniae* (36). Protoplasts were resuspended in distilled water and were lysed by several cycles of freezing and thawing. Total protein concentration was determined using the Bradford protein assay reagent (Bio-Rad).

**DNA techniques.** Preparation of plasmid DNA and electroporation-mediated transformation of *A. pyogenes* strains were performed as previously described (28). Genomic DNA from *A. pyogenes* was isolated by the method of Pospich and Neumann (40). A library of *A. pyogenes* BBR1 genomic DNA was constructed in λGEM-12, according to the manufacturer’s instructions (Promega). Methods for growth and purification of bacteriophage were es-
sentially as described by Ausubel et al. (4). DNA was prepared from bacte-
riophage as previously described (46) and was further purified using the
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 elsewhere (4). Preparation of DNA probes, DNA
hybridization, and probe detection were performed using a digoxigenin DNA
labeling and detection kit (Roche), as recommended by the manufacturer.
PCR DNA amplification was performed using Taq DNA polymerase (Fisher
Scientific) 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, with a final extension step of
72°C for 5 min.
Nucleotide sequence determination. The sequence of nanH was determined
from plasmids pJGS274 and pJGS292 and appropriate subclones using auto-
mated DNA sequencing. Sequencing was performed on both strands, crossing all
restriction sites, using KS, SK, or T7 sequencing primers or oligonucleotide
primers based on the sequence of nanH. Oligonucleotide primers were synthet-
ized by Sigma-Genosys. Sequencing reactions were performed by the DNA
Sequencing Facility at The University of Arizona, using a 377 DNA sequencer
(Applied Biosystems Inc.).
Computer sequence analysis. Nucleotide sequence data were compiled using
the Sequencher program (GeneCodes). Database searches were performed us-
ing the BlastX and BlastP algorithms (3). Sequence analysis was performed using
the suite of programs available through the Genetics Computer Group, Inc.
(University of Wisconsin). Signal sequence prediction was performed using
SignalP (38). Multiple sequence alignments were performed using CLUSTAL W
(53).
Cloning and purification of a recombinant, six-His-tagged NanH (His-NanH).
The nanH gene, lacking the coding region for the signal sequence, was amplified
from A. pyogenes BB11 genomic DNA by PCR with a 5′ primer containing an
Nhel site (5′-GGTTGCGACTGCCCCTGCGAGCAG-3′) and a 3′ primer containing a
PstI site (5′-GGCGATCCGCGCCGACATTAACG-3′) (re-
strictions sites are underlined). These primers amplified a 2.9-kb product from
bases 120 to 3009 of the plo gene. The PCR fragment was digested with Nhel-PstI
and cloned into Nhel-PstI-digested pTrcHis B (Invitrogen) to generate pJGS306.
pJGS306 encoded His-NanH, a 977-amino-acid protein comprising 963 amino
acids of the mature NanH with an N-terminal extension of 14 amino acids
coded by pTrcHis B, including a six-His sequence.
Preparation of goat antiserum to His-NanH. A female goat was immunized
with 100 g of His-NanH in a Ribi adjuvant system (Corexa) intramuscularly in
g 3Al fragment. DNA purified from
E. coli containing a 3,773-bp
JGS6 was digested with
BamHI and cloned into similarly digested pBC KS (Stratagene). Two overlapping clones encompassing the entire nanH gene region were obtained: pJGS274, containing a 3,773-bp BamHI fragment, and pJGS292, containing a 6,151-bp HindIII fragment (Fig. 1). pJGS274, but not pJGS292, conferred neuraminidase activity on the E. coli host, as determined by the MUAN filter paper assay.
RESULTS
A. pyogenes strains express neuraminidase activity. A total of 53 strains of A. pyogenes isolated from a variety of animals, including bovine, porcine, and avian species were tested for neuraminidase expression by the filter paper method. All 53 strains were positive for neuraminidase activity, indicating that this enzyme is probably expressed by all A. pyogenes isolates.
In addition, neuraminidase activity was expressed throughout the cell cycle. A. pyogenes BB11 cells, grown to an OD600 of 0.45, 1.0, or 5.2, were washed once with PBS, and an equal number of cells was applied to filter paper saturated with MUAN. All the samples fluoresced strongly, indicating that at least in vitro, neuraminidase activity was present throughout the A. pyogenes cell cycle.
Cloning and nucleotide sequence determination of nanH. During a project to clone A. pyogenes promoter sequences in the promoter-probe vector pKK232-8 (Amer sham Pharmacia Biotech), sequences homologous to the neuraminidase gene, nedA, of Micromonospora viridifaciens (45) were identified. In order to clone the entire gene, a probe was prepared from a region spanning bases 1639 to 1898 of the nanH gene and was used to probe a AEGM-12 library of BB11 genomic DNA. Several plaques hybridized strongly with the probe and were selected for further analysis. One of these, JGS6, contained an approximately 16-kb partial Sau3A1 fragment. DNA purified from JGS6 was digested with BamHI or HindIII and cloned into similarly digested pBC KS (Stratagene). Two overlapping clones encompassing the entire nanH gene region were obtained: pJGS274, containing a 3,773-bp BamHI fragment, and pJGS292, containing a 6,151-bp HindIII fragment (Fig. 1). pJGS274, but not pJGS292, conferred neuraminidase activity on the E. coli host, as determined by the MUAN filter paper assay.
The DNA sequence of the nanH gene region was deduced from these clones. There are several in-frame ATG codons at
the 5′ end of the nanH gene. The first and second ATG appear to be equal candidates for start codons, as they both have consensus ribosome-binding sites. Assuming translation from the first ATG, the 3,009-bp nanH gene encoded a protein with a predicted molecular mass of 107.3 kDa. A gram-positive signal sequence, with a cleavage site between Ala 40 and Ala 41, was predicted by SignalP (38). A putative rho independent terminator (ΔG = −17.0 kcal/mol) was identified 26 bases downstream of the nanH stop codon. No E. coli σ70-like promoter sequences were apparent upstream of the nanH open reading frame (ORF), birA, was identified, and its protein product had similarity to biotin ligase from Deinococcus radiodurans (59). Downstream sequences contained ORFs whose protein products had similarity to electron transport proteins, FixABCX, involved in nitrogen fixation in Bradyrhizobium japonicum (25, 58), birB and the putative fixABCX operon were also transcribed in the opposite direction to nanH (Fig. 1), suggesting that nanH is monocistronic.

Analysis of the primary structure of NanH. Cleavage at the predicted signal peptide sequence of NanH would result in a mature protein with a predicted molecular mass of 103.2 kDa and a pI of 6.3. NanH showed similarity to a number of bacterial neuraminidases, including those from Actinomyces viscosus (31.2% identity, 61.8% similarity), M. viridifaciens (21.4% identity, 42.3% similarity), and Bacteroides fragilis (13.7% identity, 33.4% similarity). In addition, the NanH 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 (14, 21, 43) (Fig. 2).

At the C terminus of the NanH protein there was a sequence similar to the cell wall sorting signals found in surface-expressed proteins of gram-positive bacteria (49). 33 amino acids of NanH (LVHTGTVVGLSMVAVLAA GGAIAHRRQG) consisted of an LPXTG cleavage motif (bold), a hydrophobic domain (underlined), and a positively charged stop transfer sequence (italics). However, in the case of the A. pyogenes NanH protein, the cleavage motif was LVHTG, instead of LPXTG. In addition, a Pro-rich repetitive region, which is thought to facilitate spanning of the cell wall peptidoglycan (49), was found directly upstream of the LPXTG motif.
Cloning and expression of His-NanH. To facilitate the purification of recombinant NanH from E. coli, the NanH-coding sequence, lacking the sequence for the putative signal peptide was cloned into pTrcHis B. SDS-PAGE and Coomassie brilliant blue staining of IPTG-induced cultures of DH5αMCR(pTrcHis B) (lanes 1) and DH5αMCR(pJGS306) (lanes 2) and 100 ng of purified His-NanH (lanes 3) were subjected to SDS–10% PAGE. Separated proteins were stained with Coomassie brilliant blue (A) or were transferred to nitrocellulose by Western blotting and immunostained with 1/100 E. coli-adsorbed preimmune serum (B) or 1/100 E. coli-adsorbed His-NanH antiserum (C). The positions of the molecular size standards are shown (in kilodaltons) on the left. The arrow indicates the position of His-NanH.

Determination of the prevalence of the nanH gene by DNA dot blotting. In order to determine whether nanH was present in all A. pyogenes strains, genomic DNA was prepared from 53 A. pyogenes strains and was subjected to hybridization at high stringency with a nanH-specific probe that spanned bases 1639 to 1898 of the nanH ORF. The DNA from all 53 strains hybridized strongly to the probe (data not shown), indicating that the nanH gene is present in all A. pyogenes strains.

Construction and characterization of a nanH mutant. Construction of the nanH mutant used an allelic exchange plasmid in which the nanH gene was completely replaced by an erm(X) cassette (Fig. 4). This plasmid was constructed by deletion of the 2.6-kb ClaI fragment containing the nanH gene sequences from pJGS292 (Fig. 1), resulting in the recombinant plasmid pJGS326. The 0.73-kb ClaI fragment of pJGS293, containing sequences upstream of nanH, was cloned into similarly digested pJGS326 to form pJGS354. The entire 4.3-kb HindIII insert of pJGS342 was cloned into similarly digested pHSS20 (37) to form pJGS356. The KM resistance gene in pHSS20 is functional in A. pyogenes and was used to identify the presence of recombinants which arose by a single crossover event. A 1.65-kb HindIII-BamHI fragment containing the erm(X) gene from pNG2 (50) was used as the standard incubation temperature. Purified His-NanH had a specific activity of 1.3 U/mg at pH 6.0 and 37°C.
grown overnight to an OD$_{600}$ of 3.0 to 4.0, the cells were that of the wild-type strain BBR1. BBR1 and NANH-1 were not shown). These data confirm deletion of the DNA hybridized with a pHSS20-specific (vector) probe (data specific probe was used. Neither BBR1 nor NANH-1 genomic ermA specific probe was used. Neither BBR1 nor NANH-1 genomic DNA hybridized with a pHSS20-specific (vector) probe (data not shown). These data confirm deletion of the nanH gene in BBR1 genomic DNA when an erm(X)-specific probe was used. Neither BBR1 nor NANH-1 genomic DNA hybridized with a pHSS20-specific (vector) probe (data not shown). These data confirm deletion of the nanH gene in NANH-1 by a double-crossover event.

Southern blotting of *A. pyogenes* genomic DNA digested with EcoRI revealed hybridizing bands of 1.9, 1.0, 0.9, and 0.3-kb in BBR1, when probed with a nanH-specific probe (spanning bases 120 to 3009 of the nanH ORF). No hybridizing bands were observed in a nanH mutant (NANH-1) that was probed similarly, indicating the complete loss of the nanH gene in this strain. A 2.6-kb band was apparent in EcoRI-digested NANH-1 but not BBR1 genomic DNA when an erm(X)-specific probe was used. Neither BBR1 nor NANH-1 genomic DNA hybridized with a pHSS20-specific (vector) probe (data not shown). These data confirm deletion of the nanH gene in NANH-1 by a double-crossover event.

The neuraminidase activity of NANH-1 was compared to that of the wild-type strain BBR1. BBR1 and NANH-1 were grown overnight to an OD$_{600}$ of 3.0 to 4.0, the cells were washed twice in PBS and resuspended at an OD$_{600}$ of 3.0, and the neuraminidase activity was assessed by the fluorometric assay using MUAN as a substrate. NANH-1 had fivefold less neuraminidase activity than the wild-type (Fig. 5). Therefore, insertional deletion of nanH did not result in complete loss of expression of neuraminidase activity from NANH-1, indicating that NanH is not the only neuraminidase expressed by *A. pyogenes*.

**Localization of NanH.** Whole cells, CSF, and CWE were prepared from BBR1 and NANH-1 which had been grown overnight to an OD$_{600}$ of 3.0 to 4.0. These samples were tested for neuraminidase activity with the fluorometric assay using MUAN as a substrate. The majority of neuraminidase activity was detected in whole cells and CWE from either BBR1 or NANH-1. However, BBR1 whole cells and CWE had significantly higher neuraminidase activity than whole cells and CWE from NANH-1 (Fig. 5). Some neuraminidase activity was detected in the CSF of both BBR1 and NANH-1 (Fig. 5), and this activity may have resulted from fragments of cell wall material present in the CSF. Negligible neuraminidase activity was detected in lysed protoplasts of BBR1 and NANH-1 (Fig. 5). These data indicate that the majority of NanH-specific neuraminidase activity was associated with the cell wall. In addition, the activity of the putative second neuraminidase also appeared to be cell wall associated.

**Adherence of *A. pyogenes* to epithelial cells.** It was previously demonstrated that *A. pyogenes* adhered to HeLa cells (15). We also tested the ability of BBR1 to adhere to other epithelial cell lines. BBR1 adhered to CHO, HeLa, and MDBK cells in a washing-resistant manner (Fig. 6A). However, bacterial adherence was greatest using HeLa cells (Fig. 6A), and this cell line was used in all subsequent studies.

The ability of BBR1 and NANH-1 to adhere to HeLa cells was tested. The wild-type BBR1 and nanH mutant strains adhered equally, with average adherences of 46.4 and 44.7%, respectively (Fig. 6B). In addition, the adherence of NANH-1 to CHO and MDBK cells was evaluated and was not significantly different from that of BBR1 (data not shown). These findings suggest that lack of NanH does not impair adhesion of NANH-1 to these epithelial cell lines.

In order to determine whether addition of exogenous NanH could enhance adhesion, 1 to 100 µg of purified His-NanH or an equal volume of TALON elution buffer (as a control) was added, either 1 h prior to or simultaneously with the addition of BBR1 or NANH-1 bacteria. No significant difference in the adhesion of the two strains was observed under any experimental condition (data not shown). These data indicate that addition of exogenous NanH did not increase the ability of BBR1 or NANH-1 to adhere to HeLa cells.

**DISCUSSION**

This is the first report of the cloning and sequencing of a neuraminidase gene, nanH, from *A. pyogenes*. In addition, this work provides indirect evidence that *A. pyogenes* expresses a
second neuraminidase. NanH was localized to the cell wall, and the second neuraminidase also appeared to be cell wall associated. Furthermore, we demonstrated that neuraminidase activity is expressed by all *A. pyogenes* strains tested (*n* = 53).

The *nanH* gene of *A. pyogenes* was cloned and sequenced and appeared to exist in a monocistronic operon, surrounded by the housekeeping genes *birA* and *fixABCX*. *nanH* expressed a 103.2-kDa protein with neuraminidase activity, and the NanH protein was most closely related to the neuraminidase of *A. viscosus* (61). The NanH protein contained sequences consistent with its activity as a neuraminidase, including the RIP/RLP motif and five copies of the Asp box (14, 21, 43). In addition, the finding that NanH 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 (49). However, in NanH, the cell wall anchor motif is LVHTG, which is slightly at variance with the consensus (49). However, in NanH, the cell wall anchor motif is LVHTG, which is slightly at variance with the consensus (49). Hence, nanH is expressed in *A. pyogenes* strains that have cell sorting signals where the Pro of the LPXTG motif has been replaced (B. H. Jost and S. J. Billington, unpublished data). Therefore, it appears that the *A. pyogenes* cell anchor sequence may be divergent from those seen in other bacteria.

Recombinant His-NanH protein was found to have optimal activity at pH 5.5 to 6.0 and 55°C, with no requirement for Ca²⁺ or Mg²⁺. These conditions correspond to those previously reported for a purified, native *A. pyogenes* neuraminidase (47). However, there were differences in the apparent size and cellular location of NanH (103.2 kDa, cell wall) and the purified, native *A. pyogenes* neuraminidase (50 kDa, CSF) (47). Size variation, probably as a result of specific breakdown or proteolytic cleavage, has been observed in other bacterial neuraminidases, with the truncated proteins retaining enzymatic activity. The 107-kDa *S. pneumoniae* NanA is observed as an enzymatically active 86-kDa species following purification (32). The size of the *M. viridifaciens* NedA protein varies between 41 and 68 kDa, depending on the culture conditions (45). Indeed, upon prolonged storage at 4°C, His-NanH converted to a species of 62 kDa, with no loss of specific activity (data not shown). However, it is still uncertain whether NanH is the 50-kDa neuraminidase purified from *A. pyogenes* CSF (47). NanH was localized to the cell wall of *A. pyogenes*, but NanH was also present in CSF, as NANH-1 had significantly less neuraminidase activity in the CSF than BBR1 (Fig. 5). Schaufuss and Lämmler could detect significant neuraminidase activity in the CSF of only 2 out of 42 of *A. pyogenes* strains tested, and they did not report testing whole cells or CWE for the presence of neuraminidase activity (47).

The entire nanH ORF was deleted during the construction of NANH-1, as confirmed by Southern blotting (data not shown). Significant neuraminidase activity in NANH-1 indicated the presence of a second enzyme in *A. pyogenes*. In addition, the location of the second neuraminidase also appeared to be cell wall associated, as evidenced by the retention of some neuraminidase activity in the CWE of NANH-1.

The initial event in infection by many bacteria is their attachment to mammalian cells via specific recognition structures, leading to bacterial colonization of the host. Adherence of *A. naeslundii* to both a human epithelial cell line (9) and polymorphonuclear leukocytes (44) was enhanced by pretreatment with neuraminidase. Like *A. pyogenes*, *S. pneumoniae* expresses two distinct neuraminidases, NanA (7), which is cell associated, and NanB (6), which is thought to be secreted from the cell. Neuraminidase treatment of tracheal organ cultures increased the adherence of *S. pneumoniae* (56). Furthermore, *S. pneumoniae* mutants deficient in neuraminidase activity had reduced abilities to colonize and persist in the nasopharynx (55).

It is clear that neuraminidase activity plays a role in mediating host cell adhesion of some pathogens to mucosal surfaces. In order to determine whether this was the case for *A. pyogenes*, experiments assessing the adherence of NANH-1, the neuraminidase mutant, were conducted. As previously reported, *A. pyogenes* can adhere to HeLa cells (15), and we demonstrated adherence to other epithelial cell lines, although *A. pyogenes* adhered best to HeLa cells. NANH-1 displayed no defect in adherence to HeLa cells compared with the wild-type, BBR1. If the neuraminidase activity does play a role in mediating adherence of *A. pyogenes*, it is possible that, at least under these conditions, the second neuraminidase was sufficient for maximal adherence. If this is the case, addition of exogenous His-NanH would not significantly affect adhesion to the host cell. Alternately, neuraminidase may play no role in the adhesion of *A. pyogenes* to cultured epithelial cells. The effects of neuraminidase activity may be more evident in vivo, where it may act to reduce mucus viscosity (24), assisting the adherence of *A. pyogenes* by other colonization factors, such as putative collagen- or fibronectin-binding proteins. The validity of these hypotheses is being tested in our laboratory by construction of a knockout mutant of the second neuraminidase in NANH-1 and assessment of the ability of this double mutant to adhere to host epithelial cells, both in vitro and in vivo.

ACKNOWLEDGMENTS

We thank Stefani Gilbert for construction of the AEM-12 library and Hien Trinh and Dawn Bueschel for their excellent technical assistance.

Partial support for this work was provided by USDA/NRICGP awards (97-35204-4750 and 99-35204-7818).

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Editor: J. T. Barbieri