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Cloning, Sequence, and Transcriptional Regulation of the Operon Encoding a Putative *N*-Acetylmannosamine-6-Phosphate Epimerase (*nanE*) and Sialic Acid Lyase (*nanA*) in *Clostridium perfringens*

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Clostridium perfringens **can obtain sialic acid from host tissues by the activity of sialidase enzymes on sialoglycoconjugates. After sialic acid is transported into the cell, sialic acid lyase (NanA) then catalyzes the hydrolysis of sialic acid into pyruvate and *N*-acetylmannosamine. The latter is converted for use as a biosynthetic intermediate or carbohydrate source in a pathway including an epimerase (NanE) that converts *N*-acetylmannosamine-6-phosphate to *N*-acetylglucosamine-6-phosphate. A 4.0-kb DNA fragment from *C. perfringens* NCTC 8798 that contains the *nanE* and *nanA* genes has been cloned. The identification of the *nanA* gene product as sialic acid lyase was confirmed by overexpressing the gene and measuring sialic acid lyase activity in a *nanA Escherichia coli* strain, EV78. The *nanA* gene product was also shown to restore growth to EV78 in minimal medium with sialic acid as the sole carbon source. By using Northern blot experiments, it was demonstrated that the *nanE* and *nanA* genes comprise an operon and that transcription of the operon in *C. perfringens* is inducible by the addition of sialic acid to the growth medium. The Northern blot experiments also showed that there is no catabolite repression of *nanE-nanA* transcription by glucose. With a plasmid construct containing a promoterless *cpe-gusA* gene fusion, in which β-glucuronidase activity indicated that the *gusA* gene acted as a reporter for transcription, a promoter was localized to the region upstream of the *nanE* gene. Primer extension experiments then allowed us to identify a sialic acid-inducible promoter located 30 bp upstream of the *nanE* coding sequence.**

Clostridium perfringens is a ubiquitous pathogenic bacterium. In nature, it is found in soils and sediments as well as in the intestinal tracts of animals, especially birds and mammals (21). *C. perfringens* in humans commonly causes clostridial myonecrosis (gas gangrene), acute food poisoning, and antibiotic-associated diarrhea (21); in domestic livestock, it causes a wide range of enteric diseases (15). For *C. perfringens* gangrene and enteric infections, it has been postulated that the availability of nutrients for bacterial growth is an important factor in the disease process (5). One nutrient that *C. perfringens* can readily obtain and metabolize is sialic acid.

Sialic acids comprise a family of related sugar moieties that are found throughout the body as a component of glycoproteins, gangliosides, and other sialoglycoconjugates (23). *C. perfringens* produces enzymes that release sialic acid from sialoglycoconjugates, transport it into the cell, and degrade it as a source of nutrients or biosynthetic intermediates (Fig. 1). Sialic acids are especially abundant in the intestinal tract, where they are a major constituent of mucins (23). The enzyme sialidase (or neuraminidase) is able to cleave terminal sialic acid residues from carbohydrate polymers, making them available as nutrients (5). *C. perfringens* is an unusual bacterium in that it synthesizes two different sialidase enzymes. The larger sialidase (71 kDa), NanI, is secreted from the cell and exhibits a broad substrate specificity (18). This enzyme is probably responsible for providing free sialic acid to *C. perfringens* in the intestinal tract and host tissues. *C. perfringens* also synthesizes a smaller (43-kDa) cytoplasmic sialidase, NanH (18, 19). The role of the cytoplasmic enzyme was initially puzzling, since the substrate for sialidase is usually a complex carbohydrate that cannot be transported into the cell. However, more recent work shows that NanH has a marked preference for cleaving sialic acid conjugated to short oligosaccharides (18, 20), suggesting that NanH may act on low-molecular-weight oligosaccharides that can be transported into the cell (Fig. 1).

Sialic acid lyase (NanA) is a cytoplasmic enzyme that functions to split sialic acid into pyruvate and *N*-acetylmannosamine (Fig. 1). The *C. perfringens* lyase enzyme was first purified many years ago (14), has well-characterized kinetic constants, and is commonly used as a reagent to synthesize sialic acid derivatives (5). An earlier report by Nees and Schauer demonstrated that sialic acid lyase enzyme activity, as well as sialidase enzyme activity, was induced when substrates containing sialic acid were added to the medium (13).

A small family of genes encoding proteins with high levels of amino acid sequence similarity have been found to be located near *nanA* genes in *Escherichia coli* and *Haemophilus influenzae* and in an open reading frame (ORF) unlinked to *nanA* in *Borrelia burgdorferi*. The *E. coli* gene in this family (*yhcJ*) has recently been designated as encoding an epimerase enzyme that converts *N*-acetylmannosamine-6-phosphate to *N*-acetylglucosamine-6-phosphate and has been renamed *nanE* (17). In this report, we describe the cloning, sequence analysis, and transcriptional regulation of an operon encoding the *nanE* and *nanA* gene products from *C. perfringens*. Concurrently with the

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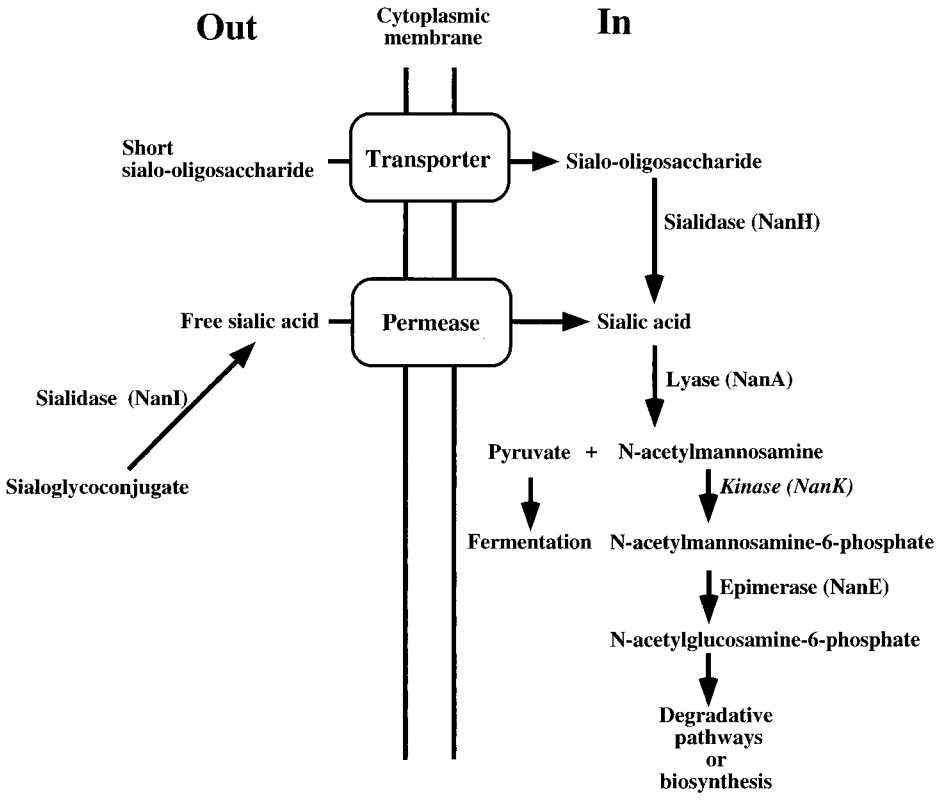

FIG. 1. Schematic diagram illustrating the proposed pathway of sialic acid metabolism in *C. perfringens*. The genes encoding each enzyme except the transporter, permease, and kinase (NanK) have been detected in *C. perfringens*.

work detailed here, Traving et al. published a report describing the cloning and sequence analysis of the *nanA* gene from *C. perfringens* A99 (26).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* JM107 and DH10B were used for the routine transformation of plasmids. Either Luria broth (LB) (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract) or M9 minimal medium [12.8 g of $Na_2HPO_4 \cdot 7H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl, and 1 g of $(NH_4)_2SO_4$ per liter] with added carbohydrate was used to grow the *E. coli* strains. To grow *C. perfringens*, anaerobic PGY medium (30 g of Proteose Peptone, 20 g of glucose, 10 g of yeast extract, and 1 g of sodium thioglycolate per liter) or PY medium (the same as PGY but lacking glucose) was prepared and stored in an anaerobic chamber (Coy Laboratory Products, Inc.), as previously described (31).

Plasmid constructs and DNA manipulations. The plasmids used in this study are listed in Table 1. Chromosomal DNA was isolated from *C. perfringens* by the method described by Mengaud et al. (11), except that the cells were incubated at 37°C for 20 min after the addition of lysozyme and 30 min after the addition of proteinase K.

In order to construct a plasmid gene bank, 10 μg of *C. perfringens* chromosomal DNA and 5 μg of pACYC184 were digested with *Eco*RI overnight at 37°C; pACYC184 was then treated with alkaline phosphatase. The digested plasmid and chromosomal DNA were electrophoresed through a 1% agarose gel. Chromosomal DNA fragments between 3 and 10 kb in length and the *Eco*RI-cut pACYC184 were purified from the gel with the Qiaex II gel extraction kit (Qiagen) according to the manufacturer's instructions. After ligation, the plasmids were transformed into *E. coli* JM107 by electroporation and plated on LB agar plates with 15 μg of tetracycline per ml. Colonies were washed off the plates with LB and stored in 15% glycerol at −80°C.

Fifty microliters of electrocompetent *E. coli* GMS343 (*manA*) cells were pulsed in a 2-mm cuvette with 1 μg of the *Eco*RI gene bank plasmid DNA and then plated on M9 medium with 15 μg of tetracycline per ml and 5 g of mannose per liter. One isolate grew above background levels but not to the same extent as the wild-type JM107 ($manA^+$) control. The transformant contained a recombinant plasmid with a 4.1-kb insert in the *Eco*RI site of pACYC184; it was called pDMW3. A 4.0-kb *Eco*RI-*Xba*I fragment from pDMW3 was subcloned into the *Eco*RI-*Xba*I sites of the plasmid pBluescript SK(−) to make pDMW9b.

The Erase-a-Base system (Promega Corp.) was utilized to make a series of nested deletions in the insert of pDMW9b. The resulting plasmids were sequenced with an Applied Biosystems, Incorporated, model 373A DNA sequencer.

For expression studies, the *nanA* gene was amplified by PCR with oligonucleotides ODMW5 (5′-CAAATA*GAATTC*GGATTTTGGGAGGAGAAAAAC-3′), which has an *Eco*RI site (shown in italics), and ODMW6 (5′-TAAGAG*CT GCAG*GCTAGTGATAAGAATTGAAATTGGAC-3′), which has a *Pst*I site. Both the PCR product (*nanA*) and pTrc99 were digested with *Eco*RI and *Pst*I and ligated together to form pDMW67.

The promoterless *cpe-gusA* gene fusion vector was created as follows. A cloned *cpe-gusA* fusion from pSM129 (31), containing 25 bases upstream of the *cpe* ATG initiation codon (including the putative ribosomal binding site) and the coding sequence for the first 13 amino acids of *Cpe*, was amplified by PCR with primers engineering 5′ *Pst*I and 3′ *Hin*dIII sites. This PCR fragment was ligated to pJIR750 at the *Pst*I and *Hin*dIII sites to make pSM212. Then, the four consecutive terminators of pRS415 (25) were amplified by PCR with primers with an *Eco*RI site at the 5′ end and a *Kpn*I site at the 3′ end to provide the proper orientation for the terminators. The PCR product was cloned into pCR2.1, which was then digested with *Eco*RI and *Kpn*I and ligated to pSM212 at the *Eco*RI and *Kpn*I sites to create plasmid pSM218.

NanA overexpression. An *E. coli nanA* Tn*10* insertion mutant, strain EV78, was obtained from Eric Vimr (27). Overnight cultures of either pDMW67 or pTrc99 in EV78 were diluted 1:50 in LB containing 100 μg of ampicillin per ml and then incubated at 37°C for 2 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and the cells were grown at 37°C until reaching stationary phase, which was approximately 2 h later. The cells were pelleted and washed with phosphate-buffered saline. Five hundred microliters of sample buffer (2% sodium dodecyl sulfate [SDS], 150 mM Tris-HCl [pH 6.8], 2% β-mercaptoethanol, 20% sucrose, and 0.05% bromophenol blue) were added to each sample prior to a 15-s sonication (model 450 Sonifier; Branson). Following sonication, the sample was boiled for 10 min and immediately cooled on ice for 2 min. Then a portion of the sample was electrophoresed through a SDS–10% polyacrylamide gel and stained with Coomassie brilliant blue, as described previously (22).

Growth of the complemented *E. coli* mutant. Overnight cultures of *E. coli* EV78(pDMW67) or EV78(pTrc99) in LB containing 100 μg of ampicillin per ml were diluted 1:100 in M9 minimal medium with 3.2 mM sialic acid and 100 μg of ampicillin per ml in sidearm flasks at 37°C. IPTG (to 0.5 mM) was added after

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
Strains		
E. coli		
JM107	*endA′ gyrA96 thi hsdR17 supE44 relA1* λ⁻ Δ(*lac-proAB*) F′ *traD36 proAB lacI*^{q} *lacZ*ΔM13	29
GMS343	*lacY1 tsx-29? glnV44* (AS)? *galK2* λ⁻ *manA4 aroD6 rpsL7000*(*str*) *mtl-1 argE3*(Oc)	16
EV78	*araD139* Δ(*argF-lac*)U169 *flbB5301 deoC1 rpsL150 relA1 ptsF25 rbsR nanA4 zgj-791*::Tn*10*	27
DH10B	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara*Δ*139* Δ(*ara leu*)*7697 galU galK* λ⁻ *rpsL endA1 nupG*	Gibco/BRL
C. perfringens		
NCTC 8798		C. Duncan
SM101	High-electroporation derivative of NCTC 8798	31
Plasmids		
pJIR750		1
pBluescript SK(−)		Stratagene
pTrc99		Pharmacia Biotech
pACYC184		3
PCR2.1		Invitrogen, Inc.
pDMW3		This study
pDMW9b		This study
pSM212		This study
pSM218		This study
pSM222		This study
pSM227		This study
pSM228		This study

8 h of growth. The optical density was then monitored with a Klett-Summerson photoelectric colorimeter until the cells reached stationary phase.

Sialic acid lyase assay in *E. coli* extracts. Overnight cultures of *E. coli* EV78 (pDMW67 or pTrc99) were diluted 1:50 in LB plus 100 μg of ampicillin per ml. The cultures were incubated at 37°C on a shaking rotor for 2.5 h. IPTG was added to a final concentration of 0.5 mM. The cultures were grown for an additional 2 h. The cells were washed twice in 50 mM potassium phosphate buffer (pH 7.0), resuspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.0), and then subjected to four rounds of sonication (model 450 Sonifier; Branson) consisting of 30 s of sonication followed by 30 s on ice. The lysed cells were centrifuged for 15 min at 15,000 × *g* to remove cell debris. The supernatant was removed and loaded on a Sephadex G-25 desalting column. Two-milliliter fractions were collected, and the protein content was measured with the Bio-Rad protein assay kit with bovine serum albumin as the standard. Either 25 or 50 μg of protein was used in the lactate dehydrogenase-coupled assay for sialic acid lyase, as previously described (30).

Primer extension and Northern blot assays. Strain NCTC 8798 was grown in 10 ml of PY medium with and without the addition of carbon sources. When the cells were at mid-logarithmic phase, the total RNA was extracted with Trizol reagent (Gibco/BRL) as described earlier (10). Primer extension analysis was carried out with 40 μg of RNA and the Promega primer extension system kit, according to the manufacturer's directions. The oligonucleotide ODMW21 (5′-CTCTTATAGCCGCTGCTCCACC-3′) annealed to the 5′ region of *nanE* mRNA. A DNA sequencing ladder was created by using ODMW21 as a primer and pDMW9b as a template, according to the United States Biochemical Co. protocol. Primer extension and sequencing products were electrophoresed through a 5% acrylamide gel and visualized by autoradiography, as previously described (10). Ten micrograms of total RNA was used for Northern blot analysis. All steps, including transferring the RNA to a nylon membrane, hybridizing with the biotinylated probe, and developing the light-emitting reaction, were based on the NEBlot Phototope and Phototope Star detection kit protocols (New England Biolabs).

Transformation of *C. perfringens* and β-glucuronidase assays. Plasmids were introduced into *C. perfringens* by electroporation, as previously described (10). β-Glucuronidase assays in *C. perfringens* whole cells were performed as previously described (10).

Nucleotide sequence accession number. The nucleotide sequence of the *nanE* and *nanA* gene region has been deposited in GenBank with the accession no. AF130859.

RESULTS

Cloning and analysis of a 4.0-kb DNA fragment containing the *C. perfringens nanE* and *nanA* genes. We undertook a search for genes involved in mannose biosynthesis in *C. perfringens*, since mannose is a component of the capsule of strain NCTC 8798 (4), the subject of this study. To isolate a potential *manA* gene of *C. perfringens* that would encode a phosphomannose isomerase, we transformed an *E. coli manA* strain, GMS343, with a plasmid library containing *Eco*RI restriction-digested chromosomal DNA from strain NCTC 8798 cloned into the *Eco*RI site of plasmid pACYC184. Transformants were selected for restoration of their ability to grow on M9 minimal medium plates with mannose as the sole carbon source for cell growth. We identified a transformant, containing pDMW3, that partially restored growth on mannose. A 4.0-kb *Eco*RI-*Xba*I fragment from pDMW3, cloned into pBluescript SK(−), was used to make pDMW9b. Preliminary sequence analysis of the insert indicated there were three complete ORFs present and two partial ORFs at the ends of the insert (Fig. 2). Two of the ORFs encode gene products similar to proteins known to be involved in sialic acid metabolism in *E. coli*: *nanE* encodes an epimerase that converts *N*-acetylmannosamine-6-phosphate to *N*-acetylglucosamine-6-phosphate (17), and *nanA* encodes sialic acid lyase (26).

The complete sequences of *nanE*, *nanA*, and a partial ORF

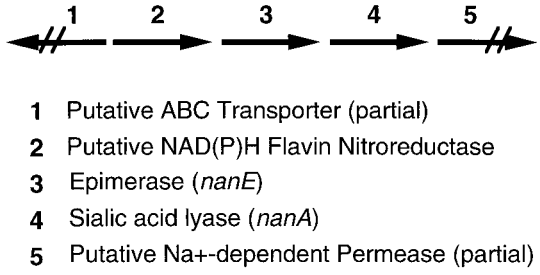

FIG. 2. Schematic diagram showing the gene arrangement in the 4.0-kb insert in pDMW9b. ABC, ATP-binding cassette.

TABLE 2. Complementation of sialic acid lyase activity and restoration of growth on sialic acid by the *C. perfringens* *nanA* gene in an *E. coli nanA* strain, EV78

Plasmid	Growth yield[a]	Sialic acid lyase activity[b]
pTrc99 (vector control)	4	Not detectable
pDMW67 (−IPTG)	37	20
pDMW67 (+IPTG)	22	329

[a] Values are Klett units at entry into stationary phase.
[b] Values are nanomoles of NADH oxidized per minute per milligram of protein.

downstream of *nanA* were determined. Concurrently with this work, a report describing the cloning and sequence analysis of the *nanA* gene from *C. perfringens* A99 and two adjacent partial ORFs was published (26); the upstream ORF is now proposed to be an ortholog of the *nanE* gene, as described in this report. There are 10 single base pair differences between the DNA sequences of the *nanA* structural genes in strains NCTC 8798 and A99 (28). Eight are silent changes, but NCTC 8798 *nanA* predicts an isoleucine at position 211 instead of the valine in strain A99 and a glutamate residue at position 278 instead of the alanine in strain A99.

The *nanE* gene encodes a protein of 221 amino acids with a predicted molecular weight of 24,167. The *C. perfringens* sequence shows the highest level of similarity (52.0% identical residues) to an ORF in *B. burgdorferi*, termed BB0644 (7), with somewhat less similarity to the NanE orthologs from *E. coli* (formerly YhcJ [2]) (40.8%) and *H. influenzae* HI0145 (39.9%) (6). The *nanA* gene of *C. perfringens* encodes a protein of 288 amino acids with a predicted molecular weight of 32,458. The NanA protein exhibits a high level of similarity to the NanA proteins of *H. influenzae* (6) (73% identical residues) and *Trichomonas vaginalis* (12) (68.5%), while it is much less similar to NanA of *E. coli* (38.4%).

A partial sequence analysis of the insert in pDMW9b upstream of *nanE* shows an ORF with a low level of sequence similarity to a putative NAD(P)H nitroreductase from *Bacillus subtilis* termed YfkO (GenBank accession no. D83967) (28) and to part of an ATP-binding cassette transporter that is transcribed divergently from the NADP(H) nitroreductase (Fig. 2) (28). Downstream of the *nanA* coding region is part of a gene (*ORF1*) that showed a low level of similarity to an Na^+-dependent permease in *Synechocystis* spp. (28).

Complementation of a *nanA* mutation in *E. coli* with the *nanA* gene of *C. perfringens*. We wanted to confirm that the *nanA* gene product in pDMW9b encodes a sialic acid lyase enzyme. Therefore, pDMW67 was transformed into the *E. coli* *nanA* strain, EV78 (27). Strain EV78 was made by transduction of the *nanA4* allele from strain EV51 into strain MC4100. The parent of EV51, strain EV36 (*nanA*$^+$), is capable of growth on sialic acid in minimal medium (27). After cell growth and the addition of 0.5 mM IPTG, a brightly staining band corresponding to a molecular mass of 33 kDa was detected in cell extracts electrophoresed through SDS-polyacrylamide gels (28), very close to the predicted molecular mass for NanA of 32.5 kDa. We next determined if the *nanA* gene expression from pDMW67 could restore lyase enzyme activity and allow growth in minimal medium with sialic acid as the sole carbon source. As seen in Table 2, pDMW67 was able to restore both sialic acid lyase enzyme activity and growth in minimal medium plus sialic acid.

Northern blot analysis indicates that *nanE* and *nanA* comprise a bicistronic operon. Since the NanE and NanA enzymes

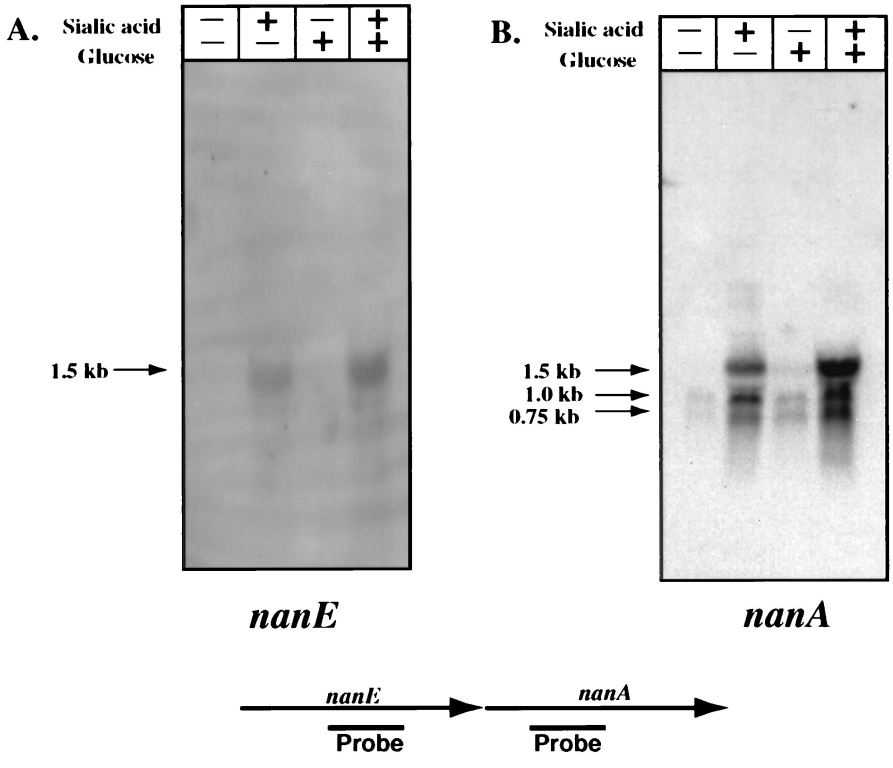

FIG. 3. (Top) Northern blot analysis of RNA from *C. perfringens* grown with different carbon sources hybridized with a *nanE*-specific probe (A) or a *nanA*-specific probe (B). (Bottom) Schematic diagram of the relative locations of the probes used for the blots shown at the top.

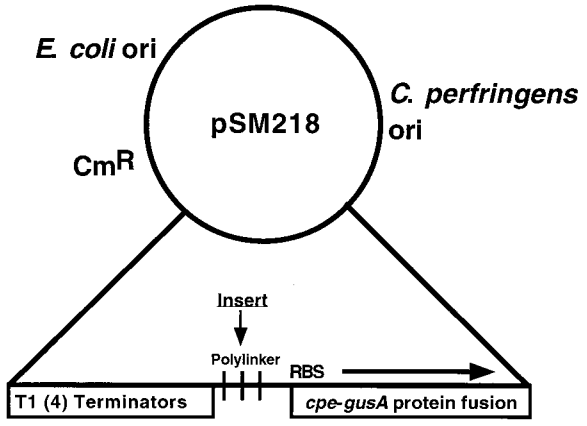

FIG. 4. Schematic diagram illustrating the relevant features of the vector used to identify promoter elements in *C. perfringens*. RBS, putative ribosome binding site; ori, origin of replication.

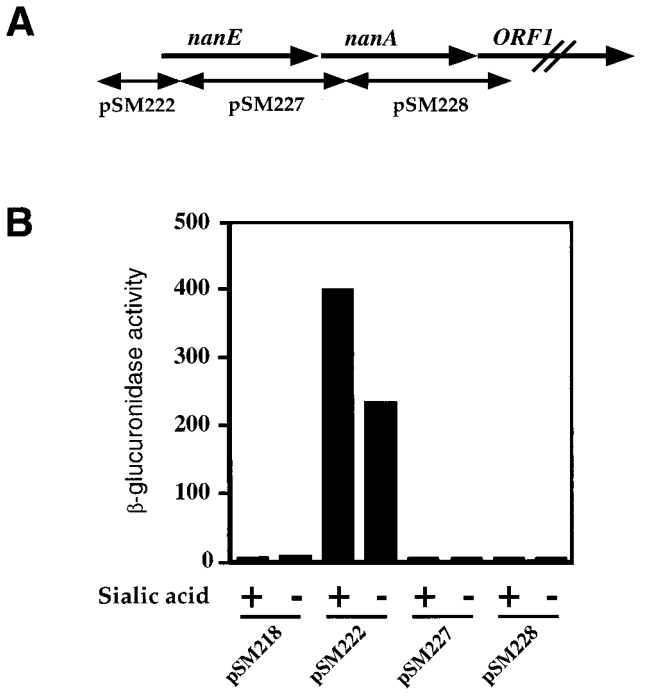

FIG. 5. Location (A) and transcriptional activity (measured as β-glucuronidase activity) (B) of DNA fragments cloned into the polylinker site of plasmid pSM218.

are involved in the same catabolic pathway, we wished to determine if they are coordinately regulated at the transcriptional level. We isolated total RNA from *C. perfringens* cells grown in PY medium with either no additional carbohydrate, 3.2 mM sialic acid, 1.4 mM glucose, or 3.2 mM sialic acid plus 1.4 mM glucose. Membranes containing this RNA were hybridized with probes internal to the *nanE* and *nanA* genes (Fig. 3). With the *nanE* probe, a band corresponding to a 1.5-kb transcript was detected when sialic acid was added to the medium, but not in its absence (Fig. 3A). The same-size band was also detected when 1.4 mM glucose was added in addition to 3.2 mM sialic acid, indicating that glucose does not have a catabolite repression effect on *nanE* transcription. Similar results were seen with a 1.5-kb RNA species when the *nanA*-specific probe was used (Fig. 3B). With the *nanA*-specific probe, two additional hybridizing bands 1.0 and 0.75 kb in length were detected under all conditions but at much higher levels in the lanes in which sialic acid was included in the medium (Fig. 3B). These shorter bands probably represent mRNA processing of the full-length *nanE-nanA* transcript, since no additional promoters could be detected downstream of the *nanE* promoter region (see next section). In addition, longer, weakly hybridizing mRNA species were detected in the lane containing sialic acid alone (Fig. 3B). Northern blot analysis with a probe specific for the putative permease gene (*ORF1*) located downstream of *nanA* (Fig. 2) failed to identify any hybridizing bands (28), which indicated that the small amount of transcription of this gene under the conditions tested was below the level of detection of the Northern blot assay.

Localization of the *nanE-nanA* promoter. We constructed a vector, pSM218 (Fig. 4), that allowed us to identify promoter elements in *C. perfringens* DNA. This vector has four tandem terminators to minimize vector-based transcription and a multiple cloning site located upstream of a promoterless *cpe-gusA* protein fusion. The ribosomal binding site and first 13 amino acids of the *cpe* gene coding region were retained to provide efficient translation (10), while the *gusA* gene (8) acted as a transcriptional reporter element. Three regions of the *nanE-nanA* operon (Fig. 5A) were placed in front of the promoterless *cpe-gusA* gene, and their abilities to initiate transcription in *C. perfringens* under inducing (PY with 3.2 mM sialic acid) and noninducing (PY with no added carbohydrate) conditions were measured (Fig. 5B). Only the fragment that included the region upstream of *nanE* exhibited transcription under the conditions tested, indicating that a promoter was located in this region. However, due to a high level of transcription under noninducing conditions, the level of transcription from the *nanE* promoter was only twofold greater when sialic acid was added to the growth medium (Fig. 5B).

Having determined that the *nanE-nanA* promoter lay upstream of the *nanE* coding region, we used primer extension experiments to locate it precisely. We detected a 5′ end located 34 bp upstream of the *nanE* coding region (Fig. 6A). In addition, this 5′ end was detected only when 3.2 mM sialic acid was added to the medium, lending additional support to the Northern blot results that indicated that *nanE-nanA* transcription is induced when sialic acid is present. A potential promoter recognition sequence was identified upstream of the start site of transcription (Fig. 6B), with five of six matches to the canonical −10 and −35 consensus *C. perfringens* housekeeping promoter recognition sequences (21).

DISCUSSION

In this study, we attempted to identify a *C. perfringens manA* locus by using a selection method based on the ability to complement a *manA* strain of *E. coli* with a plasmid gene bank. Instead, our selection led to the identification of a recombinant clone that carried *nanE* and *nanA* genes. The locations of the *nanE* and *nanA* gene products in the sialic acid metabolic pathways of *C. perfringens* are shown in Fig. 1. We do not fully understand why pDMW3 was identified in our original selection, but it could be that the epimerase and lyase enzymes affect mannose metabolic pathways in *E. coli*. We have not eliminated the possibility that the putative NAD(P)H nitroreductase or the partial coding regions played a role in the complementation experiments. The question of which gene(s) is responsible for complementing the *manA* mutation is under investigation in our laboratory.

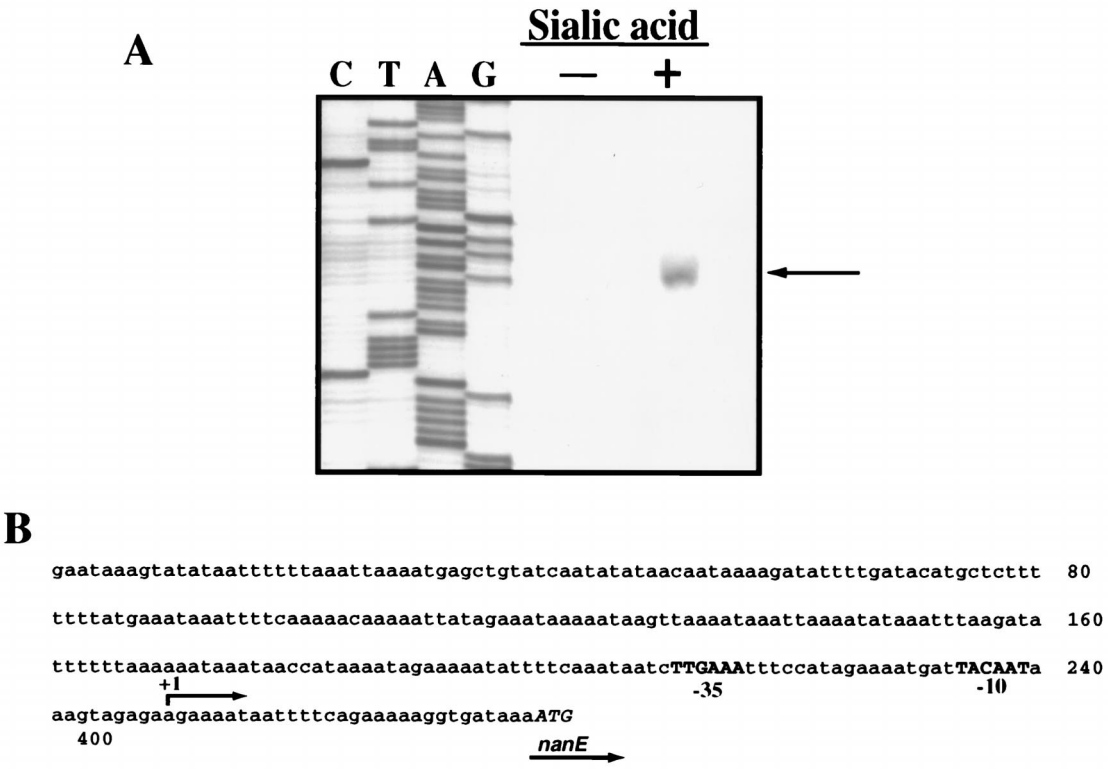

FIG. 6. (A) Primer extension results from RNA obtained from *C. perfringens* growing on PY medium with or without 3.2 mM sialic acid added. (B) Sequence of the *nanE* promoter region shown in panel A. The −10 and −35 promoter recognition sequences are shown in bold capitals. The initiator methionine codon of the *nanE* structural gene is shown in italic capitals.

We have unequivocally demonstrated that the *C. perfringens nanA* gene encodes a sialic acid lyase by both enzymatic and growth complementation studies (Table 2). An assignment for a potential function of the *nanE* gene product as an epimerase that converts *N*-acetylmannosamine-6-phosphate to *N*-acetylglucosamine-6-phosphate in *E. coli* has been made only very recently (17); we are now attempting to confirm this enzymatic activity for NanE in *C. perfringens* in our laboratory.

We propose that the *nanE* and *nanA* genes comprise an operon that is transcribed from a single promoter. This proposal is based on the results of the Northern blot experiments shown in Fig. 3, where a 1.5-kb transcript was detected with both *nanE*- and *nanA*-specific probes. If *nanE* and *nanA* are cotranscribed, this is the approximate length of transcript that would be expected. Two additional bands, 1.0 and 0.75 kb in length, were also detected with the *nanA*-specific probe, but we predict that these are the results of mRNA processing rather than alternative promoter sites. This is based on our inability, with plasmid pSM218 (Fig. 5), to detect significant levels of transcription from DNA fragments which should include any promoter that was present downstream of the start of the *nanE* coding region.

We detected low levels of probe hybridizing to longer mRNA species with the *nanA*-specific probe when sialic acid was added to the medium (Fig. 3B). However, we could not detect a hybridizing band in Northern blots with an *ORF1*-specific probe. In addition, a potential rho-independent terminator located 47 bp downstream of the *nanA* gene was identified (28). This may indicate that the longer transcripts include the upstream ORF encoding a protein with similarity to an NAD(P)H nitroreductase (Fig. 2), but at much lower levels than the *nanE-nanA* transcript. Interestingly, in *E. coli* the sialic acid transporter gene, *nanT*, is located immediately downstream of the *nanA* coding region in the chromosome (9). However, the partial sequence of *C. perfringens ORF1* does not show any significant similarity to *nanT* of *E. coli*, and this gene is apparently transcribed under different conditions than those examined in this study. Nonetheless, this does not exclude this gene from encoding a permease that can also transport sialic acid by a mechanism other than that used by the *E. coli* NanT protein.

We have also demonstrated that transcription of the *nanE-nanA* operon is inducible by sialic acid by using two methods, Northern blot analysis (Fig. 3) and primer extension experiments (Fig. 6). This data supports an earlier observation that the enzyme activity of sialic acid lyase was inducible when substrates containing sialic acid were present in the medium (13). Transcription of the *nanE-A* operon is not catabolite repressed by the presence of glucose in the medium (Fig. 3), even though excess glucose is known to repress both sporulation and amylase synthesis in *C. perfringens* (24).

The use of a multicopy vector to detect regulation at the *nanE* promoter failed to show significant levels of transcriptional regulation in the presence of sialic acid (Fig. 5); i.e., there was a considerable level of transcription even in the absence of sialic acid. This could be due to multicopy effects; e.g., a high number of plasmids may titrate away a transcriptional repressor, thereby allowing unregulated transcription at the *nanE* promoter. The *C. perfringens* replication functions of plasmid pSM218 are derived from plasmid pIP404, which is estimated to exist at 10 to 15 copies per cell in *C. perfringens* (21). As additional indirect evidence for a repressor activity, the promoter that we identified as being transcriptionally regulated by sialic acid showed matches at five of six positions

each to canonical −10 (TATAAT) and −35 (TTGACA) promoter recognition sequences for housekeeping genes in *C. perfringens*, as well as the optimum 17-bp spacer between these elements (Fig. 6B). This close match to the consensus may indicate that the promoter is transcribed unless a repressor is bound at the promoter region. Further work to identify the transcriptional regulator will help resolve this issue.

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