NanI Sialidase, CcpA and CodY Work Together to Regulate Epsilon Toxin Production by *Clostridium perfringens* Type D Strain CN3718

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Clostridium perfringens type D strains are usually associated with diseases of livestock and their virulence requires the production of epsilon toxin (ETX). We previously showed (Li et al. PLoS Pathogen. 2011, 7(12): e1002429) that BMC202, a nanI null mutant of type D strain CN3718, produces less ETX than does wild-type CN3718. The current study proved that the lower ETX production by BMC202 is due to nanI gene disruption, since both genetic and physical (NanI or sialic acid) complementation increased ETX production by BMC202. Furthermore, a sialidase inhibitor that interfered with NanI activity also reduced ETX production by wild-type CN3718. The NanI effect on ETX production was shown to involve reductions in codY and ccpA gene transcription levels in BMC202 vs. wild-type CN3718. Similarly as CodY, CcpA was found to positively control ETX production. A double codY/ccpA null mutant produced even less ETX than a codY or ccpA single null mutant. CcpA bound directly to sequences upstream of the etx or codY start codons and bioinformatics identified putative CcpA-binding cre sites immediately upstream of both the codY and etx start codons, suggesting possible direct CcpA regulatory effects. A ccpA mutation also decreased codY transcription, suggesting that CcpA effects on ETX production can be both direct and indirect, including effects on codY transcription. Collectively, these results suggest that NanI, CcpA and CodY work together to regulate ETX production, with NanI-generated sialic acid from the intestines possibly signaling type D strains to upregulate their ETX production and induce disease.
IMPORTANCE

_Clostridium perfringens_ NanI was previously shown to increase ETX binding to, and cytotoxicity for, MDCK host cells. The current study demonstrates that NanI also regulates ETX production via increased transcription of genes encoding the CodY and CcpA global regulators. Results obtained using single _ccpA_ or _codY_ null mutants and a _ccpA/codY_ double null mutant showed that _codY_ and _ccpA_ regulate ETX production independently of one another but _ccpA_ also affects _codY_ transcription. Electrophoretic mobility shift assays and bioinformatic analyses suggest that both CodY and CcpA may directly regulate _etx_ transcription. Collectively, results of this study suggest sialic acid generated by NanI from intestinal sources signals ETX-producing _C. perfringens_ strains, via CcpA and CodY, to upregulate ETX production and cause disease.
INTRODUCTION

*Clostridium perfringens* is an important cause of human and livestock infections, including many diseases originating in the intestines (1, 2). The virulence of *C. perfringens* is largely attributable to its prolific toxin-producing ability, with different toxins involved in specific diseases (1, 3, 4). By definition, type D strains must produce both alpha toxin (CPA) and epsilon toxin (ETX). ETX is a class B NIAID priority toxin since it ranks among the most potent of all bacterial toxins. This toxin is also required for type D strains to cause animal enteritis and enterotoxemia, a condition where ETX is produced in the intestines and then absorbed into the circulation so it can damage internal organs such as brain, kidney and liver (1, 5-7). During disease, an inactive ~33 kDa ETX prototoxin is initially produced and then processed to active ~27 kDa isoforms by intestinal proteases, including trypsin, chymotrypsin and other proteases, such as carboxypeptidases (8-10).

Although ETX is essential for type D disease (6), there is still relatively limited knowledge of how *C. perfringens* controls production of this toxin. Our lab did recently report that, in type D strain CN3718, ETX production does not require the VirR/VirS two component regulatory system (11). However, both the Agr-like quorum sensing (QS) system and the global gene regulator CodY were found to be necessary for CN3718 to produce natural levels of ETX (11, 12).

In Gram-positive bacteria, CodY commonly acts as a repressor of gene expression in a nutrient-rich environment (13, 14). For example, in log-phase cultures growing in rich media, CodY represses the *tcdA* and *tcdB* genes, which encode *Clostridium difficile* toxins A and B (15), as well as the *Staphylococcus aureus* *hysA* gene encoding the secreted enzyme hyaluronidase (16). Conversely, there is increasing evidence that CodY can positively regulate expression of...
some toxins made by low G/C Gram-positive bacteria, including production of anthrax toxin by
Bacillus anthracis (17), botulinum neurotoxin by Clostridium botulinum (18) and ETX by C.
perfringens (12).

Many Gram-positive bacteria produce another global regulatory protein named catabolite
control protein A or CcpA (19, 20). CcpA is a member of the Lacl/GalR family of transcriptional
regulators (19, 20) and often works together with CodY to regulate, directly or indirectly, the
expression of hundreds of genes involved in carbon and nitrogen utilization (21-23). CcpA
interacts with proteins like phosphorylated HPr, which increases its affinity for certain DNA
binding sites and results in repression or activation of gene transcription (20, 24). There is
accumulating evidence that CcpA can also be involved in the control of virulence gene
expression by several Gram-positive pathogens, including C. difficile and S. aureus (25-29). In
C. perfringens, CcpA has been shown to control expression of the enterotoxin gene (cpe) and
genes involved in type IV pilus formation and function (30, 31).

In addition to toxins, C. perfringens produces many enzymes, including three sialidases
named NanJ, NanI and NanH. The two larger sialidases, NanJ (129 kDa) and NanI (77 kDa), are
secreted from C. perfringens, while the smallest enzyme, NanH (43 kDa), is located in the
cytoplasm of log-phase cultures but can also appear extracellularly in older cultures (32, 33). A
recent study (32) found that type D strain CN3718 produces all three sialidases, with NanI being
the major secreted sialidase of this strain, as it is for other C. perfringens strains (34, 35). NanI
has an emerging potential role in pathogenesis. This sialidase was shown to increase ETX
binding and cytotoxicity for MDCK cells (32). Furthermore, NanI was demonstrated to
significantly enhance the in vitro adherence of CN3718 vegetative cells to enterocyte-like Caco-
2 cells (32).
In earlier work we noted that BMC202, an isogenic CN3718 \textit{nanI} null mutant, produces 10^5 less ETX compared to wild-type CN3718, although inactivation of \textit{nanJ} or \textit{nanH} expression did not affect production levels of this toxin (32). However, complementation was not performed in that study so it has not been distinguished whether NanI affects ETX production or if the reduced ETX production by BMC202 was instead due to a secondary mutation. Therefore, the current study first evaluated ETX production levels following genetic and physical complementation of BMC202. To begin evaluating a mechanism behind NanI effects on ETX production, transcription levels of both the \textit{codY} and \textit{ccpA} genes were compared for wild-type CN3718 vs. BMC202. Lastly, given that CodY has been shown to positively control ETX expression (12), the current study investigated whether CcpA also controls ETX expression and, if so, whether CodY and CcpA modulate \textit{etx} expression independently or in combination.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains, vectors, media and chemicals used in this study.}

Unless otherwise specified, media used for culturing \textit{C. perfringens} included FTG broth (fluid thioglycolate, Becton-Dickinson), TH broth (Todd Hewitt Broth, Becton-Dickinson) and cooked meat medium (CMM, Difco Laboratories). Media used for culturing \textit{E. coli} included LB medium (1\% Tryptone, 0.5\% Yeast Extract and 1\% NaCl, pH 7.0), RM medium (2\% Casamino Acids, 0.6\% Na$_2$HPO$_4$, 0.3\% KH$_2$PO$_4$, 0.05\% NaCl, 0.1\% NH$_4$Cl, 1mM MgCl$_2$) plus 2\% glucose. All antibiotics and chemicals used were purchased from Fisher Scientific or Sigma-Aldrich Company.

CN3718, a \textit{C. perfringens} type D livestock gastrointestinal (GI) disease strain, produces epsilon toxin (ETX) and all three (NanJ, NanI and NanH) sialidases (32, 33).
constructed mutants used in this study included: i) BMC202, an isogenic CN3718 nanI null mutant strain, ii) BMC207, an isogenic CN3718 nanJ, nanI, nanH and etx quadruple null mutant strain, and iii) BM2072, a nanI complementing strain of BMC207 (32). Another mutant used was codYko, a CN3718 codY null mutant that had been prepared in a previous study, where it was named CN3718::codY (12). Each of those null mutant and complemented strains prepared in previous studies were stored as stock cultures in CMM at -20°C (32). Plasmid pJIR750nanIcomp, which was used to prepare the nanI complementing strain BM2072, had also been prepared previously (32).

Construction and characterization of the BMC202 nanI complementing strain. Plasmid pJIR750nanIcomp, which contains the nanI gene with its own natural promoter (32), was introduced by electroporation into the BMC202 nanI null mutant to create a nanI complementing strain named BMC2022. The complementing strain was first confirmed by colony PCR assay using the internal nanI primers nanIKOF and nanIKOR (32). The PCR amplification conditions used were: 1 cycle of 95°C for 5 min and 35 cycles of 95°C for 30 s, 55°C for 40 s, and 68°C for 1 min 20 s, followed by a single extension of 68°C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide for visualization under UV light. After this PCR assay, a sialidase Western blot was performed. For this assay, a 0.2 ml aliquot from overnight FTG cultures of wild-type CN3718, BMC202 or BMC2022 was inoculated into 10 ml of TH medium and the cultures were grown at 37°C for 6 h. A 0.2 ml aliquot of those 6 h TH cultures was then transferred to 10 ml of fresh TH medium, followed by overnight (16 h) culture at 37°C (in this study, all other samples were also prepared by this standard protocol if not specifically indicated otherwise). Supernatants of the overnight TH
culture were collected and measured for sialidase activity and subjected to Western blot analysis for sialidase and ETX detection, as described later.

NanI physical complementation of the nanI null mutant BMC202. A 0.2-ml aliquot of an overnight TH culture of BMC207 (which does not produce any sialidase or ETX (32)) and BMC2072 (which is BMC207 complemented to produce NanI, but not NanJ, NanH or ETX (32)) were inoculated into 10 ml of TH medium for 2 h at 37°C. Those 2 h culture supernatants were filtered through a 0.45 µM sterile filter unit (Millipore) and sialidase activities were then determined using the method described later. BMC202 cells from an overnight TH culture were washed three times with pre-warmed PBS buffer (phosphate-buffered saline buffer, pH 7.4, Corning) and a 100-µl aliquot of those washed cells was then inoculated into 1 ml of filter-sterilized BMC207 or BMC2072 supernatants. After a 2 or 4 h culture under anaerobic conditions at 37°C, supernatants from these cultures were collected and tested for sialidase activity and ETX production, using methods described later.

Sialic acid physical complementation of the nanI null mutant BMC202. A 0.2-ml aliquot of an overnight FTG culture of BMC202 was inoculated into 10 ml of TH medium for overnight culture at 37°C. One ml of these overnight TH culture was pelleted and washed three times with warmed PBS buffer. A 100 µl aliquot of those washed cells was then inoculated into 1 ml of MEM culture medium (without FBS and Glucose, Sigma-Aldrich), supplemented with sialic acid (N-acetyl neuraminic acid, Neu5Ac, purchased from Sigma-Aldrich) at 0, 10, 1000 µg/ml. After 4 h of culture under anaerobic conditions at 37°C, supernatants from these cultures were...
collected and tested for ETX production, using methods described later. At the same time, $OD_{600}$ was checked for all cultures.

**Sialidase inhibitor effects on CN3718 ETX toxin production.** Sialidase inhibitors N-acetyl-2,3-dehydro-2-deoxy neuraminic acid (NADNA) and siastatin B (SB) were purchased from Sigma-Aldrich. The concentrations ($IC_{50}$) of these inhibitors needed for a 50% reduction in sialidase activity of CN3718 TH supernatants were determined previously (33). To assess the effects of these sialidase inhibitors on CN3718 ETX production, CN3718 cells from an overnight TH culture were washed three times with pre-warmed PBS buffer and a 100-µl aliquot of those washed cells was inoculated into 1 ml of fresh TH medium with or without different concentrations of sialidase inhibitors ($5\times$NADNA $IC_{50}$, $10\times$NADNA $IC_{50}$, $20\times$NADNA $IC_{50}$, $5\times$ SB $IC_{50}$, $10\times$SB $IC_{50}$, or $20\times$SB $IC_{50}$). After a 4 h-incubation under anaerobic conditions at 37°C, supernatants from these cultures were collected and tested for sialidase activity and ETX production using methods described below.

**Measurement of sialidase enzyme activity.** Assay of sialidase enzyme activity was performed as described previously (32). Briefly, a 20-µl aliquot of supernatant from TH cultures (prepared as described earlier) and the same volume of substrate (4 mM 5-bromo-4-chloro-3-indolyl-$\alpha$-D-N-acetylneuraminic acid, Sigma-Aldrich) were added to 60 µl of 0.05 M Tris-HCl buffer (pH 7.2) in a microtiter plate. The mixture was incubated at 37°C for 30 min and the absorbance at 595 nm was then measured using a Bio-Rad microplate reader. Sialidase enzyme activity in the samples was adjusted to fall within the linear range of the assay.
**Western blot analyses of ETX and sialidase production.** ETX and sialidase Western blots were performed as described previously (32). Briefly, supernatants from TH cultures (prepared as described earlier) were collected and mixed with 5×SDS loading buffer. After boiling for 5 min, those samples were electrophoresed on a 10% polyacrylamide gel containing SDS and transferred onto 0.45 µM nitrocellulose membranes (Bio-Rad). The membranes were blocked using Tris-buffered saline-Tween 20 (0.05%, vol/vol) with 5% (wt/vol) nonfat milk for 30 min at room temperature. The presence of ETX on the membranes was probed on the blots using anti-ETX mouse monoclonal antibody (1:1000 dilution), kindly provided by Paul Hauer. Sialidase was detected on the blots using a rabbit polyclonal antiserum against sialidases (1:1000 dilution, LifeSpin BioSciences Inc). After incubation with either i) a horseradish peroxidase-conjugated secondary anti-mouse IgG antibody (1:5000 dilution) for ETX or ii) a secondary anti-rabbit IgG antibody (1:10000 dilution) for sialidase (both purchased from Sigma-Aldrich Company) in TBS buffer containing 5% (wt/vol) nonfat dry milk, the production of ETX or sialidases was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). ETX production was quantified by Image-J software analysis of three independent Western blot results.

**ETX cytotoxicity detection.** Madin-Darby canine kidney (MDCK) epithelial cells were cultured in a 1:1 (v/v) mix of Nutrient Mixture F12 HAM (Sigma-Aldrich) and Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich) supplemented with 7.5% fetal bovine serum (Mediatech), 1% glutamine (Sigma-Aldrich) and 100 µg/ml penicillin/streptomycin (Sigma-Aldrich). A MDCK cytotoxicity assay was performed using the method described previously (32). In brief, supernatants from *C. perfringens* TH cultures were desalted and buffer exchanged.
with PBS buffer using a Millipore Ultrafiltration centrifuge tube (10,000 nominal molecular weight limit [NMWL]). A 50-µl 20×concentrated aliquot of each desalted supernatant was activated by 12.5 µg of trypsin (Sigma-Aldrich Company) for 1 h at 37°C. In order to remove trypsin activity, trypsin inhibitor (Sigma-Aldrich Company) (1:1 v/v) was added and incubated for 30 min at room temperature. These treated supernatants were then added to HBSS buffer (Hanks’ balanced salt solution, with calcium & magnesium, Mediatech Inc.), with a final volume of 1 ml. Those 1 ml samples were applied to 6-well plates containing monolayers of MDCK cells and incubated for 1 h at 37°C in a CO₂ cell incubator. ETX neutralization experiments were performed at the same time. A 50 µl aliquot of the same monoclonal antibody used for ETX Western blots was incubated for 1 h at room temperature with 1 ml of activated culture supernatants and then applied to MDCK cell monolayers for 1 h at 37°C in a CO₂ cell incubator.

Cytotoxicity was then measured using the LDH Cytotoxicity Detection kit, which was purchased from Roche Scientific Company. The same trypsin/trypsin inhibitor (T/TI) mix without ETX or 50 µl of ETX-neutralizing monoclonal antibody in 1 ml of buffer alone were used as negative controls for this cytotoxicity assay.

Construction of a CN3718 ccpA null mutant, a CN3718 codY and a ccpA double null mutant and reversed mutant strains. Using the Clostridium-modified TargetTron gene knockout system (36, 37), the ccpA gene was mutated by an intron insertion in CN3718. Since inserted introns do not encode antibiotic resistance in this system, the same approach was used to mutate the ccpA gene in the isogenic codY null mutant codYko, creating a codY and ccpA double null mutant. The 900-bp intron was targeted and inserted between nucleotides 540 and 541 of
ccpA ORF in the sense orientation. Primers used for preparing the intron were: 540|541s-IBS (5'-AAAAAGCTTATAATTATCCTTAATGAACAACATAGTGCGCCCAGATAGGGTG-3'), 540|541s-EBS1d (5'-CAGATTGTACAAATGTTGATAACAGATAAGTCAACATAGCT-AACTTACCTTTCTTGT-3') and 540|541s-EBS2 (5'-TGAACGCAAGTTTCTAATT-TCGGTTTTTCATCCGATAGGAAAGGTCTTCT-3').

The Targetron plasmid (named pJIR750ccpAi) containing this 350-bp PCR product was electroporated into either CN3718 or the codYko mutant. Transformants were selected on BHI agar plates containing 15 μg/ml of chloramphenicol and then PCR-screened for an intron-disrupted ccpA gene by using primers ccpAkoF (5'-CTTAAGGAAAAATGGTTGATGGT -3') and ccpAkoR (5'-GACCATTTTCAAGCATTGCTA-3'). The PCR reactions included 1 μl of each pair of primers (at a 0.5 μM final concentration), 1 μl of purified DNA template (100 ng), and 25 μl of 2×Taq Mixture (NEB), which were all mixed together before ddH2O was added to reach a total volume of 50 μl. The reaction mixtures were placed in a thermal cycler (Techne) and subjected to the following amplification conditions: 1 cycle of 95°C for 2 min, 35 cycles of 95°C for 30 s, 55°C for 40 s, and 68°C for 1 min 20 s, and a single extension of 68°C for 5 min. PCR products were then electrophoresed on a 2% agarose gel, which was stained with ethidium bromide.

The ccpA null mutant (named ccpAko) and the codY/ccpA double null mutant (named Dko) were each grown in FTG medium without antibiotics for 10-15 days, with daily subculturing, to cure the intron-carrying donor plasmid pJIR750ccpAi. As described previously (37), null mutants retransformed with an Ltr-encoding plasmid (in this case pJIR750ccpAi) can be induced to reverse their intron-induced mutation or mutations. For this purpose, the reversed mutants (named ccpArev or Dkorev) were grown at 30°C, which resulted in i) partial LtrA-
mediated splicing removal of the intron from \textit{ccpA} mRNA, thus restoring some CcpA expression in \textit{ccpArev}, or ii) partial LtrA-mediated splicing removal of introns from both \textit{ccpA} mRNA and \textit{codY} mRNA, thus simultaneously restoring some CcpA and CodY expression in the Dkorev strain. Splicing removal of introns from disrupted mRNA in the reversed mutants was confirmed by RT-PCR, as described below.

**Southern blot hybridization analyses.** To demonstrate the presence of single (\textit{ccpA} null mutant) or double (\textit{ccpA/codY} double null mutant) intron insertions, DNA was isolated from the \textit{C. perfringens} strains using the MasterPure\textsuperscript{TM} Gram Positive DNA Purification Kit (Epicentre). An aliquot of each purified DNA (3 µg) was then digested with EcoRI overnight at 37°C and electrophoresed on a 1% agarose gel. After alkali transfer to a nylon membrane (Roche), the blot was hybridized with a digoxigenin-labeled, intron-specific probe as described previously (37). This intron-specific probe was prepared with a PCR DIG-labeling kit (Roche). DIG detection reagents were purchased from Roche Applied Science. CSPD substrate (Roche) was used for detection of hybridized probes according to the manufacturer’s instructions.

**RNA extraction, reverse transcription PCR (RT-PCR) and quantitative reverse transcription PCR (qRT-PCR).** As described previously, saturated phenol (Fisher Scientific) was used to extract RNA from pelleted cells of 3 h or 6 h \textit{C. perfringens} cultures grown in TH medium. All RNA samples were treated using a DNase kit (TURBO DNA-free\textsuperscript{TM}, Ambion by Life Technologies) to remove DNA contamination. DNase inhibitor (included in the same kit) was then added to stop DNase activity. The purified RNA was quantified by absorbance at 260 nm and stored in a −80°C freezer.
RT-PCRs were performed on 100 ng of purified RNA samples using the AccessQuick RT-PCR system (Promega). Primers (ccpAkoF and ccpAkoR) were targeted to sequences located upstream and downstream of the inserted intron, when present, in the ccpA ORF. RT-PCR of 16sRNA served as a positive control (primers for 16sRNA were used as described before (12)). qRT-PCR was performed using the Applied Biosystems One-step RT-PCR with SYBR green, and a real-time PCR instrument (Applied Biosystems) with a 96-well reaction module. Each qRT-PCR was performed in triplicate with 20 ng of total RNA and a 500 nM concentration of each primer. The reaction conditions used were described previously (12). All qRT-PCR primers were designed from the IDT website. The qRT-PCR primers used to amplify the 16sRNA gene and etx sequences were published before (12). The primers used for ccpA qRT-PCR were qccpAF (5’-ACACATAGAACAAAGAACTGTAGGT-3’) and qccpAR (5’- TACGTCCTCAGCACCTCTAA -3’); the primers used for qRT-PCR amplification of codY transcripts were qcodYF (5’- GTGCTACAATAGTTGGGATGGA-3’) and qcodYR (5’- CCAATAGCTAATTGAACCAC TGC -3’). After qRT-PCR, the relative quantitation of mRNA expression was normalized to the constitutive expression of the housekeeping 16sRNA gene and calculated by the comparative threshold cycle (Ct) (2−ΔΔCt) method (12).

**Growth curve measurements for wild-type CN3718 and null mutant strains.** A 0.2-ml aliquot of an overnight FTG culture of CN3718 or each null mutant strain was transferred to 10 ml of TH medium. After a 6-h culture at 37°C, a 0.2 ml aliquot of each TH starter culture was transferred to 10 ml of fresh TH medium, which was cultured overnight (~16 h) at 37°C (for growth curve comparisons of wild-type CN3718, BMC202 and BMC2022) or 30°C (for growth curve comparisons of wild-type CN3718, ccpAko, Dko and the reversed mutant strains ccpArev
A 0.2-ml aliquot of each TH overnight culture was then transferred to 10 ml of TH medium and those cultures were grown at 37°C or 30°C, as specified; at every two hours until 10 h, the OD<sub>600</sub> of each culture was determined using a Bio-Rad Smart Spec Plus spectrophotometer.

Purification of recombinant CcpA His<sub>6</sub>-tagged protein and use of that protein in an electrophoretic mobility shift assay (EMSA). To purify a His<sub>6</sub>-tagged recombinant CcpA protein (rCcpA-His<sub>6</sub>), the ccpA gene was PCR-amplified from CN3718 DNA using specific primers ccpAhis-pBAD-F (5’-TCCGG AATTCTGAGGAGATGATTAAATGGCTGCTTCAA TTAAAG-3’) and ccpAhis-pBAD-R (5’-ATGAGGTACCTTAATGATGATGATGATGTTTACAGCTATCTTTCTATTA-3’). These two primers introduced EcoRI and KpnI restriction sites at both ends of the ccpA-coding sequence and a His<sub>6</sub>-tag at the C-terminal end to assist purification. The PCR product was cloned into pBAD30 (15) that had been digested with EcoRI and KpnI, creating the CcpA His<sub>6</sub>-tag protein vector named pBADccpA. pBADccpA was then introduced by transformation into the E.coli KS272 ara<sup>−</sup> strain (15). Cells containing pBADccpA were grown at 37°C in RM medium containing 100 µg ml<sup>−1</sup> of ampicillin until an OD<sub>600</sub> of 0.5 was reached. Production of rCcpA-His<sub>6</sub> was induced for 4 h by adding 0.2% L-arabinose (Sigma-Aldrich). The His<sub>6</sub>-tagged rCcpA was purified from cell lysates as described previously (12). The purity of rCcpA-His<sub>6</sub> was >90% based upon Coomassie blue G250 staining of an SDS gel and the identity of the purified protein was confirmed by Western blot analysis using a mouse monoclonal antibody against polyhistidine (Sigma-Aldrich).

The EMSA was performed as described previously (12). Briefly, DNA fragments were mixed with increasing amounts of rCcpA-His<sub>6</sub> protein in 10-µl of reaction buffer that contained
20 mM Tris-Cl (pH 8.0), 50mM sodium glutamate, 10mM MgCl₂, 5mM EDTA, 0.05% (vol/vol) Nonidet P-40 (Sigma-Aldrich) and 5% (vol/vol) glycerol. After incubation of those mixtures for 30 min at room temperature, the binding reaction mixtures were added to 2 µl of 6×EMSA gel-loading solution (Electrophoretic Mobility Shift Assay kit; Invitrogen). Those mixtures were then loaded onto a 6% nondenaturing Tris-borate-EDTA (TBE) polyacrylamide gel that had been pre-run at 4°C for 60 min at 100 V in 0.5×TBE. After loading the samples, the gel was electrophoresed for 80 min at 200 V under the same conditions. The gel was stained for 20 min with SYBR Green and the stained gel was then imaged using a Typhoon 9400 variable-mode imager (Amersham Biosciences), with fluorescence emission set to detect the SYBR Green label using the green laser with a wavelength of 532 nm.

DNA fragments used for EMSA included the sequences upstream of the codY and etx start codons. The primers 1016gsF and 1016gsR were used to amplify sequences upstream of the etx start codon (12). The DNA fragment corresponding to the 606-kb sequence present immediately upstream of the codY ORF was PCR amplified from CN3718 using the primers codYupF (5’-ACTCCATTAAAGGAAGACATA GAAAAGG-3’) and codYupR (5’-TGTCGACATTATATCCTCCTCG-3’). As a control for specificity, 340-bp DNA fragment was PCR amplified from internal codY sequences using the primers codYkoF and codYkoR, which have been described before (12). All DNA fragments were gel purified before mixing with the rCcpA-His₆ protein.

Bioinformatics. For the following bioinformatics analyses, the upstream regions of codY (600 bp) and cepA (664 bp) from C. perfringens strain CN3718 were sequenced by the Genomics Research Core at the University of Pittsburgh. These sequences were deposited in GenBank with
the following accession numbers: KT323923 (codY upstream region) and KT323924 (ccpA upstream region). The upstream region of etx from this strain was previously accessioned (JN543539.1).

To evaluate if the etx and codY genes have sequences corresponding to putative upstream cre site motifs that may allow for direct binding and regulation by CcpA in C. perfringens strain CN3718, the following bioinformatics analyses were performed. Using the online Multiple Em for Motif Elicitation software (MEME Suite 4.10.1; http://meme-suite.org) (38), the regions 600 bp upstream of etx and 500 bp upstream of codY (corresponding to the regions used for EMSA assays) were entered into the MEME software alongside 52 putative cre box/CcpA-binding sequences from C. perfringens ATCC 13124 as curated on the RegPrecise v3.3 website ((39); http://regprecise.lbl.gov). The parameters were as follows: zero or one occurrence per sequence on the given strand of DNA with a width of 6 to 50 nt.

For determination of whether sequences corresponding to putative CodY-binding sites are present upstream of etx and ccpA in CN3718, MEME analysis was performed using the 600 bp upstream of etx and the 500 bp upstream of ccpA. These sequences were entered into the MEME software along with the overall CodY consensus sequence “AATTTTCWGAAAAATT,” the C. difficile CodY consensus sequence “TTYWRAATWTTWRAATWTTY,” and 55 putative CodY-binding sequences as identified in C. difficile (15). The parameters for these analyses were as follows: any number of occurrences per sequence on the given strand of DNA with a width of 6 to 50 nt.

Statistical Analyses. All statistical analyses were performed with GraphPad Prism 6, and using the ordinary one-way ANOVA.
RESULTS

Genetic complementation of a NanI sialidase null mutation restores ETX production. In our previous study (32) evaluating the relationship between sialidases and ETX action, ETX production was compared between wild-type CN3718 and several isogenic sialidase null mutants. Interestingly, the BMC202 nanI null mutant showed decreased ETX production compared to wild-type CN3718. However, isogenic nanJ and nanH single null mutants both still exhibited similar ETX production levels as the parent strain (32).

Those results suggested that NanI production might enhance epsilon toxin production but an alternative explanation could have been that BMC202 has some unrecognized secondary mutation. To rigorously evaluate whether NanI expression affects ETX production levels, it was necessary to determine if NanI genetic and physical complementation can increase ETX production by BMC202. To initiate this work, a nanI complementing strain named BMC2022 was prepared in the current study. To prepare BMC2022, BMC202 was transformed using the pJIR750nanIcomp plasmid, which contains the CN3718 nanI ORF, 500 bp of upstream sequence and 300 bp of downstream sequence cloned into the C. perfringens/E. coli shuttle plasmid pJIR750. PCR detected the presence of the wild-type nanI ORF in the BMC2022 complemented strain (data not shown).

Before analyzing ETX expression, a sialidase Western blot and a sialidase activity assay were performed for overnight TH culture supernatants from wild-type CN3718, BMC202 and BMC2022 (Figs. 1A and 1B). The results confirmed a previous report (32) that BMC202 does not produce NanI and that its exosialidase activity is significantly decreased compared to...
CN3718. In contrast to BMC202, the BMC2022 complemented strain exhibited an increase in both NanI production and sialidase activity (Figs. 1A and 1B).

An ETX Western blot was then performed using the same supernatants from TH cultures of CN3718, BMC202 and BMC2022. The results of this analysis (Fig. 1C) showed that ETX production decreased significantly (about 50%) for this nanI null mutant, as reported previously (32). Importantly, ETX production was recovered by nanI complementation of the BMC202 mutant (Fig. 1C). This decreased ETX production by the isogenic nanI null mutant was not due to lower numbers of bacteria because the wild-type strain, mutant and complemented strain all grew virtually the same (Fig. 1D).

We then evaluated the potential biological significance of this altered ETX production by comparing the MDCK cell cytotoxic effects of supernatants from CN3718, BMC202 and BMC2022 (Fig. 1E). After trypsin treatment to activate their ETX, and subsequent trypsin neutralization with trypsin inhibitor, supernatants from cultures of CN3718 or the complementing strain BMC2022 caused nearly 2-fold more cytotoxicity than did similarly treated supernatants from a culture of the BMC202 mutant. Confirming the involvement of ETX in the cytotoxicity induced by culture supernatants of CN3718 and its derivatives, preincubation of an ETX-neutralizing monoclonal antibody with activated supernatants reduced the cytotoxicity to <10% on MDCK cells (Fig. 1E). In the absence of culture supernatants, treatment with buffer containing only trypsin and trypsin inhibitor (T/TI), or only ETX-neutralizing monoclonal antibody, caused virtually no cytotoxicity in MDCK cells (Fig. 1E).

NanI can physically complement ETX production by BMC202. Fig. 1 results predicted that it should also be possible to use NanI to physically complement ETX production by BMC202. Our
first attempt to demonstrate this physical complementation involved addition of a commercial NanI enzyme preparation (Roche) to a BMC202 culture. After incubation at 37°C, the culture supernatants were assayed for sialidase activity and ETX production, but negative results were observed for ETX production (not shown). However, follow-up studies then determined that incubation of this NanI enzyme preparation with highly purified ETX caused ETX degradation (not shown), indicating contamination of the commercial NanI enzyme preparation with an ETX-degrading protease.

Therefore, to demonstrate NanI physical complementation of ETX production, two previously constructed mutant strains were used (Fig. 2A), including BMC207 (which does not produce any sialidase or ETX) and BMC2072 (BMC207 complemented to produce NanI). After 2 h culture in TH medium, the supernatants were collected and filter sterilized. Sialidase activity present in those sterile BMC2072 culture supernatants was similar to that of supernatant from wild-type CN3718, while little sialidase activity was detected in BMC207 supernatants (Fig. 2B).

When washed BMC202 cells were added to sterile BMC207 or BMC2072 supernatants prepared as described above, and then cultured for 2 h or 4 h at 37°C, substantially more ETX production was observed using BMC2072 supernatant versus BMC207 supernatant. This enhancement was noted using even 2 h BMC2072 supernatants, with this effect increasing further using 4 h supernatants (Fig. 2C). Furthermore, when the sialidase activity in 2 h or 4 h supernatants were quantified, the results indicated that the sialidase activity in BMC2072(202) supernatant were much higher than the sialidase activity in BMC207(202) supernatant. Using 4 h BMC207(202) supernatant some sialidase activity was noted (Fig. 2B), likely because BMC202
still produces NanJ, which is a minor exosialidase present in supernatants of log-phase cultures of CN3718 and these CN3718-derivative strains (32, 33).

**Sialic acids can signal the upregulation of ETX production by BMC202.** Results shown in Figures 1 and 2 might predict that sialic acid should signal the nanI null mutant BMC202 to upregulate its ETX production. To test this hypothesis, sialic acid was added to BMC202 cultures in MEM without glucose or serum. After 4 h of anaerobic incubation, an ETX Western blot detected a sialic acid dose-related increase in ETX production by BMC202 (Fig. 3A). This sialic acid-induced increase in ETX production was not due to enhanced growth of BMC202 under these culture conditions (Fig. 3B).

**Effects of sialidase inhibitors on ETX production.** As a final confirmation that NanI affects ETX production by wild-type CN3718, we asked whether ETX production by this strain can be reduced by the addition of sialidase inhibitors to TH cultures. Two sialidase inhibitors, NADNA and SB, were used in these experiments since our previous study had demonstrated that these two inhibitors sharply reduce sialidase activity when added to CN3718 TH overnight culture supernatants (33); that previous finding was confirmed in the current study (Fig. 4A and 4B).

To evaluate whether these inhibitors can affect ETX production by CN3718, NADNA or SB were added at the time of inoculation and the culture was then incubated for 4 h at 37°C under anaerobic conditions (Fig. 4C), after which the supernatant sialidase activity and ETX production were measured in those cultures. The presence of the SB inhibitor substantially reduced culture sialidase activity, as expected (Fig. 4D). Addition of SB also reduced ETX production to near BMC202 production levels (Fig. 4E and Fig. 1B).
Surprisingly, the presence of the NADNA sialidase inhibitor did not inhibit sialidase activity in CN3718 cultures (Fig. 4D), even when used at a very high (20×IC₅₀) dose (data not shown). In agreement with our hypothesis that NanI levels affect ETX production levels, the CN3718 cultures grown in the presence of NADNA, but still possessing strong sialidase activity, made the same amounts of ETX as CN3718 grown in the absence of any sialidase inhibitor (Fig. 4F).

Effects of a nanI null mutation on etx, codY and ccpA transcript levels. Gram-positive bacteria often sense changes in nutrient availability using global regulators, such as CcpA and CodY (20). Therefore, we postulated that, in the nanI null mutant of CN3718, there is decreased availability of NanI-generated end products, such as sialic acids, relative to the parent strain and this difference affects transcription of global regulator genes such as ccpA or codY that are important for responding to changes in intracellular metabolite pools and influence numerous adaptive responses, including virulence. Consistent with this hypothesis, our previous study (12) demonstrated that CodY positively regulates ETX expression by CN3718, although the influence of NanI on CodY regulation was not explored in that previous study.

To test our hypothesis, qRT-PCR was performed to compare transcription of the etx, ccpA and codY genes in wild-type CN3718, BMC202 or BMC2022 strains grown for 3 h or 6 h in TH broth. These analyses (Fig. 5) detected lower etx transcription levels in the BMC202 nanI null mutant strain compared to the wild-type and complemented strains, demonstrating that the lower ETX production noted in Fig. 1 for this strain involves transcriptional regulation. Comparing the 3 h and 6 h results, etx gene transcription levels in the BMC202 nanI mutant did not substantially change.
Transcription of both the *codY* and *ccpA* genes also significantly decreased in the BMC202 mutant and those defects could be complemented. Unlike *etx* transcription by the BMC202 mutant, *codY* and *ccpA* transcript levels decreased further between a 3 h and 6 h incubation (Fig. 5).

**Construction and characterization of a CN3718 ccpA null mutant and reversed mutant strain.** The Figure 5 results were consistent with NanI effects on ETX production involving two global regulators, i.e. CodY and CcpA. As mentioned, we previously demonstrated (12) that CodY, which regulates ETX production, can bind to sequences upstream of the *etx* start codon, suggesting direct regulation of ETX production. To begin testing whether CcpA might also control ETX production, the type D strain CN3718 was used for preparation of an isogenic *ccpA* null mutant, which was constructed using *Clostridium*-modified Targetron technology (36, 37).

Using DNA from wild-type CN3718, internal *ccpA* PCR primers specifically amplified the PCR product of ~400 bp (Fig. 6A). However, using DNA from the *ccpA* null mutant strain, the same primers amplified a PCR product of 1300 bp, which is consistent with insertion of the 900 bp intron into the CN3718 *ccpA* ORF (Fig 6A). Since PCR analysis demonstrated the presence of an intron insertion in the wild-type *ccpA* ORF, the intron delivery plasmid pJIR750ccpAi was cured from the *ccpA* null mutant strain, now named ccpAko. A Southern blot performed with an intron-specific probe demonstrated the presence of only a single intron insertion in this *ccpA* mutant. No probe hybridization to wild-type DNA was detected, as expected (Fig. 6B).

Complementation to restore ETX production was not achieved by transforming the pJIR750 shuttle plasmid containing a cloned *ccpA* gene into ccpAko, possibly due to multi-copy
plasmid effects (data not shown). However, previous studies (37, 40-42) have shown that
mutations from group II introns inserted in the sense orientation can be partially reversed at the
mRNA level by growth at 30°C in the presence of an LtrA-encoding plasmid, such as
pJIR750ccpAi, with the frequency of this reversion varying between different intron insertions.
Therefore, pJIR750ccpAi was reintroduced into ccpAko, creating strain ccpArev, in order for
LtrA to remove by splicing under permissive 30°C conditions, the sense-oriented intron from
some ccpA mRNA, thus restoring a functional ccpA transcript and CcpA production. As shown
in Fig. 6C, the presence of wild-type ccpA transcripts (no intron insertion) was confirmed in both
the ccpArev and wild-type strains by RT-PCR, although the expression level was weaker for the
ccpArev strain. This was expected since splicing-induced intron removal from mRNA is only
partially efficient (37). This effect was not due to loss of the inserted intron from the ccpA gene
in ccpArev since the same PCR used in Fig. 6A still amplified only the 1.3 kb product from the
ccpA null mutant after overnight culture at 30°C (not shown).

When vegetative growth was compared amongst wild-type CN3718, ccpAko null mutant
and ccpArev strains in TH medium at 30°C (as needed later to allow ccpA mutation reversal for
ccpArev), the ccpA null mutant grew similarly to the wild-type and reversed mutant strains up to
3 h of culture and slightly more slowly thereafter (Fig. 6D). When ETX production by CN3718,
ccpAko and ccpArev in TH medium was investigated under this 3 h growth condition, the ccpA
null mutant produced substantially less ETX compared to the wild-type parent or the reversed
mutant strains (Fig. 6E). This sharp reduction in ETX production by the ccpA null mutant strains
was also noted at overnight time points (Fig. 6E), where growth differences between strains were
also relatively minor.
Two qRT-PCR assays were performed for transcription of the etx and codY genes in wild-type CN3718, ccpAko or ccpArev strains grown in TH medium at 30°C for 3 h. Results of these qRT-PCR assays demonstrated that the transcription levels of both the etx and codY genes were substantially decreased in ccpAko compared to the wild-type and ccpArev strains (Fig. 6F). Under the same culture conditions, qRT-PCR was performed for transcription of the ccpA gene in wild-type CN3718, codYko or codYcomp strains. Results of this qRT-PCR experiment showed that transcription levels of the ccpA gene were the same in all three tested strains (data not shown).

Construction and characterization of a CN3718 ccpA and codY double null mutant and a reversed mutant strain. To investigate whether CodY and CcpA control etx independently or in combination, a ccpA and codY double null mutant strain (named Dko) was prepared. Since our introns do not encode antibiotic resistance markers, the same intron-carrying plasmid used to prepare ccpAko could again be used to knock-out the ccpA gene in the codYko strain. Using internal ccpA PCR primers, DNA from ccpAko and Dko specifically amplified larger PCR products than were obtained using DNA from the wild-type or the codYko strains, consistent with insertion of an intron into their ccpA gene (Fig. 7A). Furthermore, internal codY PCR primers amplified larger PCR products from codYko and Dko compared to the products amplified by these primers using DNA from the wild-type or the ccpAko strains (Fig. 7A). Collectively, these PCR results demonstrated that Dko contains intron insertions into both the ccpA and codY genes. As expected, a Southern blot performed using an intron-specific probe showed the presence of only a single intron insertion in the ccpAko and codYko strains, while
two intron insertions were detected in the Dko strain (Fig. 7B). There was no probe hybridization
to DNA from the wild-type DNA, as also expected (Fig. 7B).

A reversed mutant strain was also prepared for Dko since Targetron technology allows
simultaneous reversion of multiple intron-disrupted genes present in a single bacterial cell. As
shown in Fig. 7C, RT-PCR analysis confirmed the presence of mRNA transcripts lacking intron
insertions from the *ccpA* and *codY* genes in this Dkorev strain. This effect was not due to loss of
the inserted intron from the *ccpA* and *codY* genes since the same PCRs used in Fig. 7A still
amplified a 1.3 kb product from Dkorev cultured overnight at 30°C (not shown). As controls for
the RT-PCR, *ccpA* mRNA transcription was not detected in the ccpAko strain and *codY* mRNA
transcription was not detected in the codYko strain (Fig. 7C). Neither *ccpA* nor *codY* mRNA
transcription was detected in the Dko strain (Fig. 7C).

When the CN3718, Dko or Dkorev strains were cultured in TH medium at 30°C to allow
mutation reversal in Dkorev, Dko entered the stationary phase only slightly earlier than CN3718
or the reversed mutant strain (Fig. 7D). ETX Western blot analyses revealed that the Dko strain
produced significantly decreased amounts of ETX production compared with the parent and
reversed mutant strains in the overnight culture (Fig. 7E). This effect was not due to a secondary
mutation in the Dko mutant, since reversal of the mutations substantially restored ETX
production. Notably, the Dko strain produced even less ETX than did either the *codY* or *ccpA*
single mutants.

A qRT-PCR was also performed for transcription of the *etx* gene in wild-type CN3718,
ccpAko, codYko, Dko or Dkorev strains grown in TH medium at 30°C for 3 h. Results of this
qRT-PCR experiment first confirmed the previous results that the transcription levels of the *etx*
gene were significantly decreased in ccpAko or codYko compared to the wild-type (Fig. 7F).
Under the same culture conditions, qRT-PCR was performed for transcription of the \( etx \) gene in Dko or Dkorev strains. Results of this qRT-PCR experiment showed that transcription levels of the \( etx \) gene were significantly decreased in the Dko strain, even lower than the single knock out strains, and that Dkorev had a partially recovered \( etx \) transcription level (Fig. 7F).

**Evidence that CcpA may both directly and indirectly regulate ETX production.** In a previous study (12), we found that CodY directly binds to sequences upstream of the \( etx \) start codon. Therefore the current study examined whether CcpA might also regulate ETX production by a direct effect where CcpA binds to sequences upstream of the \( etx \) start codon, thus controlling ETX production. For this purpose, an EMSA gel mobility shift assay was performed and this analysis demonstrated the ability of recombinant CcpA (rCcpA) to bind to a DNA fragment containing sequences present upstream of the \( etx \) start codon (Fig. 8).

The Fig. 6 experiments had shown that \( codY \) transcription levels decreased in the isogenic \( ccpA \) null mutant strain compared to the wild-type parent or complementing strains. Therefore, EMSA was performed to evaluate whether purified rCcpA can also bind to sequences upstream of the \( codY \) start codon. Results shown in Fig. 8, are consistent with CcpA directly affecting CodY production since CcpA can bind to sequences immediately upstream of the \( codY \) start codon.

The rCcpA binding to \( codY \) or \( etx \) upstream sequences was specific since no shift in mobility was observed using a control DNA fragment, even at higher rCcpA concentration.

**Bioinformatics identification of putative cre sites (CcpA-binding motifs) and CodY binding sequences upstream of \( etx \), \( ccpA \) and \( codY \).** The Fig. 8 EMSA analyses indicated that purified
rCcpA can directly bind to DNA fragments containing sequences present upstream of the etx and codY start codons. Therefore, MEME bioinformatics analyses were performed to determine if etx and codY genes might contain upstream sequences corresponding to putative catabolite repression element (cre) boxes, since those cre sites are permissive for CcpA binding and lead to subsequent transcriptional regulation of these genes (43). Comparing the region upstream of the codY and etx start codons with 52 predicted C. perfringens cre sites (39), revealed a shared motif (Fig. 9A) with a total E-value of 6.9e-70. Upstream of the codY gene (88 bp), the sequence “ATGTTAATATTTAAA” was identified (P-value; 6.29e-4), which strongly matches the consensus predicted cre box motif in C. perfringens. Upstream of the etx start codon (30 bp) we found the sequence “TAGAAATTATATTA” (P-value; 2.05e-3), which is also a match with the consensus predicted cre box motif in C. perfringens (Fig. 9A). The presence immediately upstream of the codY and etx start codons of sequences with strong homology to cre sites is consistent with the Fig. 8 EMSA data showing binding to DNA fragments containing these putative cre boxes. Taken together these bioinformatics analyses offer important further support for the possibility that CcpA binds to these cre site sequences and then positively regulates transcription of the codY and etx genes. Interestingly, there are also putative cre sites upstream of the ccpA start codon, opening the possibility that CcpA self-regulates its production (Fig. 9A).

EMSA assays performed in our previous study (12) had demonstrated that CodY binds to regions upstream of the etx start codon. To determine if there might be putative CodY-binding sites upstream of the etx start codon, MEME analysis was performed using overall CodY-binding consensus sequences, C. difficile CodY-binding consensus sequences, and 55 putative CodY-binding sites from C. difficile (15). This analysis identified a conserved motif (Fig. 9B) with an overall E-value 8.2e-50. Two potential CodY binding sites were identified upstream of the etx
start codon, with one site located 370 bp upstream of the ATG start
(“AAATCTAAGAAAAAT”; P-value 5.68e-4) and the other site present 36 bp upstream of the
ATG start (“AAAAATAGAAAAATT”; P-value 4.01e-4) (Fig. 9B).

Additionally, we searched for the CodY-binding motif upstream of the ccpA gene, and
identified the putative CodY-binding site “TCATTTTAGAAAATT” 225 bp upstream of the
ccpA ATG start site (P-value 1.59e-4). However, it appears that this sequence is not involved in
CcpA production under the culture conditions assayed since interruption of codY had no effect
on ccpA expression (data not shown).

DISCUSSION

Previous studies showed that inactivation of the nanI gene in C. perfringens type A strain
13 increased production of alpha-toxin and perfringolysin O (35). However, the current study has
established that NanI has an opposite effect on ETX production by type D strain CN3718.
Specifically, introduction of a nanI null mutation resulted in significantly lower ETX production
and this effect was reversible by complementation, either genetically with a plasmid carrying the
wild-type nanI gene or physically by supplementation with NanI or sialic acid added into culture
media.

The extent of NanI-induced regulation of ETX production observed in this study was ~2-
3 fold. While this regulatory effect is relatively modest, its biological significance was
demonstrated using ETX-induced cytotoxicity assays. The 2-3 fold increase in ETX production
induced by NanI may be important with respect to pathogenesis since many type B and D strains
produce quite small amounts of ETX (7, 44)
The current study then explored how NanI affects the regulation of ETX production. This phenotype was not simply attributable to NanI effects on growth since the NanI null mutant grew similarly to wild-type CN3718 under the culture conditions used. This result does not preclude NanI contributions to growth under other culture conditions, as recently reported (45).

An alternative possibility to growth enhancement was that NanI action could increase ETX production by inducing signaling via global transcription regulators such as CodY and CcpA. Consistent with this possibility, our group had reported previously that CodY increases ETX production (12). That previous observation (12) provided the first evidence for positive CodY regulation of toxin gene expression in a pathogenic \textit{Clostridium} spp and represented one of relatively few examples at that time of positive gene expression regulation by CodY. However, since our report, CodY was also shown to positively regulate botulinum neurotoxin gene expression by \textit{C. botulinum} (18). The current study now implicates CodY in NanI-mediated positive control of ETX production since a nanI null mutation decreases codY gene transcription levels, coincident with less ETX production. However, our ETX Western blot results would indicate that NanI effects on codY transcription account for only a portion of the overall CodY regulatory effects on ETX production.

Previous studies reported that CodY often acts additively or antagonistically with another regulator named CcpA to regulate carbon and nitrogen metabolism (21-23). CcpA can mediate repression of virulence gene expression as well as carbon catabolism genes expression in Gram-positive bacteria including some \textit{Clostridium} spp. For example, CcpA represses xylose utilization by \textit{Clostridium acetobutylicum} (46) and also represses toxin TcdA and TcdB production by \textit{C. difficile} (25, 26). Similarly, a previous study showed that a \textit{C. perfringens} mutant deficient in CcpA production exhibits significantly increased PLC and PFO production.
(47). However, CcpA is known to positively regulate some Clostridium spp. gene expression. For example, CcpA positively regulates expression of the key solventogenic operon sol in C. acetobutylicum and is necessary for efficient sporulation of C. acetobutylicum and C. perfringens (30, 46, 48). It is also necessary for CPE production by C. perfringens sporulating cultures (30).

The current study demonstrated that NanI not only affects codY gene transcription levels but also impacts ccpA gene transcription levels. Using a ccpA null mutant, it was then determined that CcpA positively regulates ETX production. This finding, to our knowledge, provides the first evidence for CcpA-mediated positive regulation of toxin gene expression in vegetative cultures of C. perfringens. It is again notable from ETX Western blots that abolishing CcpA production has a stronger effect on ETX production than does abolishing NanI production, indicating that NanI effects account for only a portion of CcpA regulation of ETX production levels.

Introducing a double ccpA and codY null mutation into CN3718 further decreased ETX production. Taken together, our results indicate that CodY and CcpA can regulate ETX production individually, but can also act together in combination. An electrophoretic mobility shift assay showed that both proteins can bind upstream of the etx start codon in CN3718, consistent with their directly regulating etx expression, alone or in combination. Important further support for this possibility was the bioinformatics-based identification of a putative CcpA-binding cre box and CodY binding sites directly upstream of the etx start codon.

The involvement of both CodY and CcpA in positive regulation of ETX production by C. perfringens is similar to the regulation of ackA (acetate kinase) gene expression by Bacillus subtilis. While CcpA and CodY alone are able to activate expression of the ackA gene, CodY and CcpA can act additively to cause full activation of the ackA promoter (21). A putative model for
the transcriptional activation of the ackA gene by CcpA and CodY has been proposed, whereby
these two proteins, along with other proteins, form a transcription initiation complex at the ackA
promoter that increases ackA gene transcription (49). The impact of NanI on C. perfringens ETX
expression via CodY and CcpA co-regulation appears to fit this ackA regulation model but this
requires future experimental verification.

Interestingly, CcpA was also shown to modulate codY expression. To our knowledge,
this is the first report of CcpA control of CodY expression in a Gram-positive bacterium.
Consistent with direct CcpA regulation of codY expression, rCcpA was demonstrated to bind to
sequences upstream of the codY start codon. The binding of purified rCcpA to sequences
upstream of both codY and etx start codons was relatively weak, which is fully consistent with
the CcpA literature (46, 48, 50), where interaction of CcpA with other proteins (such as
phosphorylated HPr) was shown to greatly increase DNA binding affinity. The possible role of
protein partners such as phosphorylated Hpr in mediating CcpA regulation of etx and codY
expression will require further study.

Relative to wild-type CN3718, transcription levels of both the codY and ccpA regulatory
genes dropped substantially in the nanI null mutant strain, particularly with longer culture times.
That may reflect, in part, a response to decreased signaling by NanI action. However, under the
culture conditions used in this study, the growth rate of the nanI null mutant remained similar to
that of wild-type CN3718, possibly due to other compensatory pathways. For example, in the
absence of NanI, C. perfringens may switch to growth using proteins encoded by genes that are
normally repressed by the CodY and CcpA regulatory systems.

Considering these findings and results from other studies, a model can be proposed for
NanI effects on the regulation of ETX production (Fig. 9C). In this model, NanI generates free
sialic acid from glycoproteins or glycolipids. A recent study (45) strongly suggests that *C. perfringens* contains the genes necessary for sialic acid uptake and metabolism that would generate cytoplasmic carbohydrates (such as fructose 1,6 bisphosphate) that are known to enhance the function of CcpA regulatory complexes. The activated CcpA complex would then bind to *cre* sites present upstream of *codY*, *ccpA* and *etx* start codons, resulting in increased ETX and CodY production and, perhaps, increased CcpA production. The increase in CodY would also affect ETX production, alone and in combination with CcpA, by resulting in more CodY binding to CodY boxes located upstream of the *etx* start codon.

Further studies are needed to test the proposed model but the results of this study already offer an intriguing potential insight towards understanding intestinal disease caused by ETX-producing *C. perfringens* strains. Since many sialic acid-containing glycolipids and glycoproteins are present on host cell surfaces and in intestinal mucus, NanI-mediated signaling may alert type B and D strains to their presence in the intestines. This signaling would result in upregulated ETX production, which can then promote disease. Host death from ETX-induced enterotoxemia, or nutrients released by ETX-induced diarrhea, may then provide an abundant food source for further growth of these *C. perfringens* strains.

Lastly, NanI sialidase contributes to *C. perfringens* adhesion to Caco-2 cells, causes an upregulation in ETX toxin production, and enhances toxin action on host cells (32). These findings suggested that sialidase inhibitors may represent a potential therapeutic approach since they can block *C. perfringens* sialidase activity in supernatants and reduce colonization of Caco-2 cells (32). However, results from the current study indicated that not all sialidase inhibitors may be useful as therapeutics. Compared to SB, NADNA is an even more effective inhibitor of sialidase activity in culture supernatants, yet NADNA has little effect on the sialidase activity in
C. perfringens cultures. Consequently, NADNA has no effect on ETX production and also causes a weaker inhibition of Caco-2 cell adherence (34). Why NADNA has limited function in vivo requires future investigation. Another important question that needs to be addressed is whether NanI is important for C. perfringens attachment, growth, toxin production and action in vivo.

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FIGURE LEGENDS

Figure 1. NanI sialidase null mutant BMC202 complementation and characterization. Panel A, Western blot of sialidases present in overnight TH medium culture supernatants of wild-type CN3718, the nanI-null mutant BMC202 and the complemented strain BMC2022. Marker size is shown at left. Note that NanH is 43 kDa, NanI is 77 kDa and NanJ is 129 kDa (32). Panel B, Sialidase activities were measured in supernatants from overnight TH cultures of wild-type CN3718, BMC202 and BMC2022. *, P<0.005 (Ordinary One-way ANOVA, compared to wild-type strain); All experiments were repeated three times, and mean values are shown. The error bars indicate standard deviation. Panel C, Left panel shows the Western blot analysis for ETX production by overnight TH medium culture supernatants of wild-type CN3718, the nanI-null mutant BMC202 and the complemented strain BMC2022. The immunoreactive protein size is at
left. Right panel shows the quantitative analysis of ETX production by CN3718 (this value was set as 100%), BMC202 and BMC2022 strains. The band intensities of the Western blot were compared by Image J analysis. *, P<0.005 (Ordinary One-way ANOVA, compared to wild-type strain). All experiments were repeated three times, and mean values are shown. The error bars indicate standard deviation. Panel D, Growth curve of CN3718, BMC202 and BMC2022 in TH medium at 37°C. A representative result from three repetitions is shown. Panel E, MDCK cell cytotoxicity after treatment with trypsin-activated supernatant from overnight TH culture supernatant of CN3718, BMC202 or BMC2022. “+ antiETX” indicates the culture supernatant was preincubated with an ETX-neutralizing monoclonal antibody. Controls shown included buffer with T/TI (trypsin and trypsin inhibitor alone, no culture supernatant), or antiETX (monoclonal antibody alone). *, P<0.005 (Ordinary One-way ANOVA, compared to wild-type strain). All experiments were repeated three times, and mean values are shown. The error bars indicate standard deviation.

**Figure 2. Physical complementation of nanI null mutant strain BMC202.** Panel A, Physical complementation protocol. BMC2072 or BMC207 were cultured in TH medium at 37°C for 2 h and the supernatants were then collected. BMC202 washed cells (100µl) were inoculated into 1 ml of these supernatants and anaerobically cultured in GasPak jars for 2 h or 4 h. The supernatants were then collected to detect sialidase activities and for ETX Western blotting. Panel B, Sialidase activities were measured in the supernatants from 2 h TH culture supernatants of BMC2072 or BMC207, as well as in the same supernatants that were inoculated with BMC202 washed cells and then cultured for 2 h or 4 h. All experiments were repeated three times, and mean values are shown. The error bars indicate standard deviation. Panel C, A
representative Western blot (from three independent experiments) for ETX production by BMC202 washed cells cultured in BMC2072 supernatant versus BMC207 supernatant for 2 h or 4 h.

**Figure 3. Sialic acid can signal for increased ETX production.** Panel A, Top: Western blot showing ETX production by BMC202 grown for 4 h in MEM (no glucose or serum) in the presence or absence of specified amounts of purified sialic acid (SA). The size (35 kDa) of the immunoreactive protein, which matches the molecular mass of ETX, is indicated to the left of the blot. Panel A, Bottom: the relative band intensities of the Western blot compared by Image J analysis. The experiment was repeated three times and mean band intensities are shown. The error bars indicate standard deviation. *, P<0.005 (Ordinary One-way ANOVA, compared to wild-type strain). Panel B, Comparison of BMC202 culture OD₆₀₀ after a 4 h anaerobic incubation at 37°C in the presence or absence of sialic acid. Mean results ± standard deviation are shown for three repetitions.

**Figure 4. Effects of sialidase inhibitors (SB or NADNA) on sialidase activity and ETX production.** Inhibition of wild-type CN3718 TH culture supernatant sialidase activity by sialidase inhibitors. Panel A, Experimental protocol. Panel B, Sialidase activities of wild-type CN3718 supernatant (Mock), as well as the same supernatant supplemented with 5×IC₅₀, of SB or NADNA. All experiments were repeated three times, and mean values are shown. The error bars indicate standard deviation. Inhibition of CN3718 TH culture sialidase activity when the culture medium supplied with sialidase inhibitors. Panel C, Experimental procedure. Panel D, Sialidase activities of wild-type CN3718 wash cell cultured in TH medium, which was
supplemented with 5×IC₅₀ of SB or NADNA, for 4 h at 37°C. All experiments were repeated three times, and mean values are shown. The error bars indicate standard deviation. Panel E, Western blot of ETX production when TH medium supplied with SB (5×IC₅₀, 10×IC₅₀ or 20×IC₅₀). Protein size marker is on the right. The band intensities of the Western blot were compared by Image J analysis. All experiments were repeated three times, a representative result is shown. Panel F, Western blot of ETX production when TH medium supplied with NADNA (5×IC₅₀, 10×IC₅₀ or 20×IC₅₀). Protein size marker is on the right. The band intensities of the Western blot were compared by Image J analysis. All experiments were repeated three times, shown is a representative graph of three independent experiments.

**Figure 5. Quantitative RT-PCR analyses of etx, codY or ccpA transcription for 3 h or 6 h TH culture of CN3718, BMC202 or BMC2022.** Panel A, 3-h TH medium cultures; Panel B, 6-h TH medium cultures. Average C_T values were normalized to the housekeeping 16s RNA gene, and the fold differences were calculated using the comparative C_T method (2⁻ΔΔC_T). Values of each bar indicate the calculated fold change relative to wild-type CN3718. All experiments were repeated three times, and mean values are shown. The error bars indicate standard deviation.

**Figure 6. ccpA null mutant strain preparation and characterization.** Panel A, PCR confirmation of the construction of an isogenic ccpA null mutant strain. Using DNA isolated from CN3718, a PCR using internal ccpA gene primers amplified the expected product of about 400 bp. Using DNA template isolated from ccpAko, which has a 900 bp inton insertion in ccpA gene, the same PCR assay amplified a larger product of 1300 bp. Line 1 is a 100 bp molecular ruler. Panel B, intron-specific Southern blot hybridization with DNA from wild-type or ccpAko
strain. DNA from each stain was digested with EcoRI and electrophoresed on a 1% agarose gel. Size of DNA fragment in kb is indicated. Panel C, RT-PCR analysis for ccpA transcription of CN3718, ccpAko or ccpArev strain. Upper panel shows transcription of housekeeping gene 16s RNA; lower panel shows transcription of ccpA gene. Line 1 is a 100 bp molecular ruler. Panel D, post-inoculation change in optical density (OD₆₀₀) for cultures of wild-type CN3718, the ccpA null mutant ccpAko and reversed mutant strain ccpArev growing in TH medium at 30°C. Panel E, ETX Western blot analysis of CN3718, ccpAko or ccpArev strains grown in TH medium culture at 30°C for 3 h or O/N. Panel F, Quantitative RT-PCR analyses of etx or codY transcription for a 3 h TH culture of CN3718, ccpAko or ccpArev strain. Average Ct values were normalized to the housekeeping 16s RNA, and the fold differences were calculated using the comparative Ct method (2⁻ΔΔCT). Values of each bar indicate the calculated fold change relative to wild-type CN3718. All experiments were repeated three times and mean values are shown. The error bars indicate standard deviation.

**Figure 7. ccpA and codY double null mutant strain preparation and characterization.** Panel A, PCR confirmation of the construction of an isogenic ccpA, codY or double null mutant strain. The same PCR assay amplified a larger product for the null mutant strain. Upper panel shows the codY PCR product amplified using DNA from CN3718, codYko, ccpAko or Dko. Only codYko and that Dko DNA amplified a larger band. Lower panel shows the ccpA PCR product amplified from the same strains, with both ccpAko and Dko DNA supporting amplification of a larger band. Line 5 is a 100 bp molecular ruler. Panel B, Intron-specific Southern blot hybridization with DNA from wild-type, ccpAko, codYko or Dko strains. DNA from each stain was digested with EcoRI and electrophoresed on a 1% agarose gel. Size of DNA fragment in kb is shown at
In wild-type CN3718, no intron-specific band was detected. One intron-specific band was detected in the codY or ccpA single null mutant strains and two bands in the Dko strain. Panel C, RT-PCR analysis for codY (upper panel) or ccpA (lower panel) transcription of CN3718, ccpAko, codYko, Dko or Dkorev strains. Line 1 is a 100 bp molecular ruler. “CN3718 DNA” shows the PCR product of chromosomal DNA using the same primers as for the RT-PCR. Panel D, post-inoculation changes in optical density (OD600) for cultures of wild-type CN3718, Dko and the reversed complementing strain Dkorev when grown in TH medium at 30°C. Panel E, ETX Western blot analysis of CN3718, ccpAko, codYko, Dko or Dkorev strains in TH medium cultured overnight at 30°C. Panel F, Quantitative RT-PCR analyses of etx transcription in 3 h TH culture of CN3718, ccpAko, codYko, Dko or Dkorev strains. Average CT values were normalized to the housekeeping 16s RNA, and the fold differences were calculated using the comparative C_T method \(2^{-\Delta\Delta CT}\). Values of each bar indicate the calculated fold change relative to wild-type CN3718. All experiments were repeated three times, and mean values are shown. The error bars indicate standard deviation.

**Figure 8. Gel mobility shift analysis.** EMSA gel mobility shift assay for the binding of rCcpA to the sequences upstream of the etx ORF (etxup) and codY ORF (codYup). A negative control DNA fragment that corresponded to the internal codY gene sequences was used. Each DNA fragment was incubated with increasing concentrations of rCcpA.

**Figure 9. Model for the interplay between NanI, CodY, and CcpA in the regulation of codY and etx.** Panel A, MEME analysis was performed using regions upstream of the ccpA, codY and etx genes, as well as predicted CcpA-binding cre sites of C. perfringens, in order to identify an
overall *C. perfringens* cre site motif consensus sequence and putative cre sites upstream of these 880 genes. Panel B, Parallel MEME analysis was performed using the region upstream of the *etx* 881 gene and putative CodY-binding sites from *C. difficile* in order to identify putative CodY- 882 binding sites upstream of *etx*. Each putative cre box or CodY-binding site is shown along with 883 the distance upstream of the *etx* or *codY* “ATG” start codon and p-values corresponding to the 884 strength of consensus matching is shown. Panel C, Model for NanI-induction of ETX production. 885 See text in Discussion for an explanation. Note that steps up to the generation of sialic acid by 886 NanI occur extracellularly but the remainder of the model involves intracellular events. Red 887 boxes depict putative cre sites, while blue boxes indicate putative CodY binding sites. “?” 888 indicates other potential, but unknown, regulators of *codY* or *ccpA* transcription.

**REFERENCE**


(A) BMC2072 (Nal^t, ETX^-) or BMC207 (Nal^t, ETX^-) 

\[ \text{TH medium, 2 h 37°C} \]

100 µl washed O/N BMC202 (Nal^t, ETX^+)

\[ \text{supernatant} \]

37°C, 2 h and 4 h

Detect ETX production and sialidase activity

(B) OD595

![Graph showing OD595 values](image)

(C) 2 h 4 h

![Image showing protein bands](image)
<table>
<thead>
<tr>
<th>0 μM</th>
<th>codY</th>
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<tr>
<td>0 μM</td>
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<tr>
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