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The VirSR Two-Component Signal Transduction System Regulates NetB Toxin Production in *Clostridium perfringens*▽

Jackie K. Cheung,[1] Anthony L. Keyburn,[2,3,4] Glen P. Carter,[1] Anouk L. Lanckriet,[5] Filip Van Immerseel,[5] Robert J. Moore,[1,2,3,4] and Julian I. Rood[1,2,4]*

Department of Microbiology[1] and Australian Research Council Centre of Excellence in Structural and Functional Microbial Genomics,[2] Monash University, Clayton, Vic 3800, Australia; CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Vic 3220, Australia[3]; Australian Poultry Cooperative Research Centre, Armidale, NSW 2315, Australia[4]; and Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium[5]

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Clostridium perfringens **causes several diseases in domestic livestock, including necrotic enteritis in chickens, which is of concern to the poultry industry due to its health implications and associated economic cost. The novel pore-forming toxin NetB is a critical virulence factor in the pathogenesis of this disease. In this study, we have examined the regulation of NetB toxin production. In *C. perfringens*, the quorum sensing-dependent VirSR two-component signal transduction system regulates genes encoding several toxins and extracellular enzymes. Analysis of the sequence upstream of the *netB* gene revealed the presence of potential DNA binding sites, or VirR boxes, that are recognized by the VirR response regulator. *In vitro* binding experiments showed that purified VirR was able to recognize and bind to these *netB*-associated VirR boxes. Furthermore, using a reporter gene assay, the *netB* VirR boxes were shown to be functional. Mutation of the *virR* gene in two avian *C. perfringens* strains was shown to significantly reduce the production of the NetB toxin; culture supernatants derived from these strains were no longer cytotoxic to Leghorn male hepatoma cells. Complementation with the *virRS* operon restored the toxin phenotypes to wild type. The results also showed that the VirSR two-component system regulates the expression of *netB* at the level of transcription. We postulate that in the gastrointestinal tract of infected birds, NetB production is upregulated when the population of *C. perfringens* cells reaches a threshold level that leads to activation of the VirSR system.**

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming rod that secretes many toxins and extracellular enzymes. It is widespread in the environment and in the gastrointestinal tracts of both humans and animals. In addition to human diseases, such as gas gangrene and food poisoning (33, 34), *C. perfringens* is also the causative agent of several enterotoxemic diseases of animals, including necrotic enteritis (40, 41). In chickens, necrotic enteritis presents as either an acute clinical or subclinical disease and has a significant global economic impact; it is estimated to cost the international poultry industry approximately US$2 billion each year (43, 45). The acute form of this disease is believed to involve several predisposing factors (44) that allow *C. perfringens* type A to multiply to high numbers in the small intestine, and it results in the concomitant elaboration of extracellular toxins that damage the intestines. Tissue disruption ranges from macroscopic lesions on the small intestinal mucosa to extensive necrosis of the mucosal surface in severe cases (45).

Early studies suggested that alpha-toxin was the major virulence factor involved in the pathogenesis of necrotic enteritis (1–3). Although this toxin has been shown to be critical in human gas gangrene (4), recent virulence tests with chickens using defined chromosomal alpha-toxin mutants clearly demonstrated that it was not essential for necrotic enteritis (20). A novel toxin, NetB, was identified and shown to be a critical virulence factor in this disease. *C. perfringens netB* mutants were unable to induce disease in chickens, which was reversed by complementation with the wild-type *netB* gene (19). Both native and recombinant forms of NetB were cytotoxic to chicken Leghorn male hepatoma (LMH) cells *in vitro* but not to several other cell lines. A gas gangrene strain that does not produce NetB but produces alpha-toxin and perfringolysin O (PFO) was not toxic for LMH cells (19).

At present, no information is available regarding how the production of this key toxin is regulated. In the human gas gangrene isolate, strain 13, the production of alpha-toxin and perfringolysin O, the toxins implicated in gas gangrene, is regulated by the VirSR two-component signal transduction system, which comprises the VirS sensor histidine kinase and its cognate response regulator, VirR (9, 22, 38). Since its initial identification, it has become the best-characterized two-component system in the clostridia. It is a global regulatory network, since it not only regulates toxin production but controls the expression of housekeeping genes (7, 18, 29, 39) and genes encoding several regulatory RNA molecules (7, 31, 39).

The VirSR phosphorelay appears to be mediated by quorum sensing (30). In this system, it is proposed that threshold concentrations of a peptide that is synthesized and secreted by the product of the *agrBD* genes are detected by the VirS sensor histidine kinase. Autophosphorylation of VirS is followed by phosphotransfer to VirR (9). The VirR response regulator then recognizes and binds independently to two imperfect directly repeated sequences, called the VirR boxes, located up-

* Corresponding author. Mailing address: Department of Microbiology, Monash University, Clayton, Vic 3800, Australia. Phone: 61 3 9902 9157. Fax: 61 3 9902 9222. E-mail: julian.rood@monash.edu.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
E. coli strains		
DH5α	F$^-$ ϕ80Δ *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdr17*(r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1*	Life Technologies
TOP10	F$^-$ *mcr*A Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lac*ZΔM15 Δ*lac*X74 *nupG recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1* λ$^-$	Invitrogen
BL21(DE3)(C43)	F′ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)(C43)	24
C. perfringens strains		
JIR325	13 Nal^r Rif^r	22
EHE-NE18	Australian chicken necrotic enteritis isolate	20
NE18Δ*netB1*	EHE-NE18 Δ*netB*::*catP*	19
56	Belgian chicken necrotic enteritis isolate	14
JIR4228	JIR325 Ω Tn*916* (Δ*pfoR pfoA colA luxS*)	5
JIR12377	EHE-NE18 *virR* Ω targetron	This study
JIR12382	56 *virR* Ω targetron	This study
Plasmids		
pMTL540F	Clostridial expression vector	15
pJIR750	*C. perfringens-E. coli* shuttle vector, Cm^r	6
pJIR750ai	pJIR750Ωalpha-toxin targetron	8
pJIR751	*C. perfringens-E. coli* shuttle vector, Em^r	6
pJIR1456	*C. perfringens-E. coli* shuttle vector with *oriT*, Cm^r	21
pJIR1877	pUC18(EcoRI/HindII) harboring the product of PCR with UP/JRP372 (EcoRI/HindIII; 1.0 kb) containing *virR* and its promoter	23
pJIR1897	pJIR750 Ω (HindIII/EcoRI: pJIR1877, 1.0 kb, *virR*)	9
pJIR2233	pJIR750 (HindIII/BamHI) harboring the *virR* promoter (HindIII/BamHI: pJIR1877, 0.214 kb)	This study
pJIR2373	*pfoA* reporter gene vector, Em^r	10
pJIR2422	pJIR2373(EcoRV) harboring annealed complementary primers 17443/17444 (34 bp) (wild-type *pfoA* VirR boxes)	10
pJIR2529	pJIR2233(BamHI/SacI) harboring the *virRS* operon (BamHI/SacI; 2.1 kb)	This study
pJIR3464	pJIR3754 (FspI/SpeI) harboring the replication region from pMTL540F (NheI/BstZ171; 1.73 kb)	This study
pJIR3562	pJIR3756 (MluI) harboring P$_{fd}$*ermB*-RAM (MluI; 1.23 kb)	This study
pJIR3566	Clostridial targetron vector derived from pJIR750ai, contains *ermB*-RAM and *lacZ*α, Cm^r	This study
pJIR3575	pJIR2373(EcoRV) harboring annealed complementary primers JRP4051/JRP4052 (34 bp) (*netB* VirR boxes)	This study
pJIR3608	Group II intron of pJIR3566 retargeted to the 546/547 site of the *virR* gene	This study
pJIR3753	pJIR750ai (HindIII/SphI) harboring P*gdh* promoter region (HindIII/SphI; 207 bp)	This study
pJIR3754	pJIR3753 (XbaI/SphI) harboring a PCR product containing the *oriT* of pJIR1456	This study
pJIR3756	Clostridial vector harboring SOE PCR product (SalI/XbaI; 321 bp) containing the ferredoxin promoter (P$_{fd}$) fused to the *ermB*-RAM (P$_{fd}$*ermB*-RAM)	This study

stream of the target genes (12). We have previously shown that VirR directly activates transcription of the perfringolysin O structural gene, *pfoA*, and that the maintenance of the integrity, spatial organization, and helical phasing of the VirR boxes is crucial for optimal levels of perfringolysin O production (10, 12). VirR boxes are located upstream of several other genes in three different strains of *C. perfringens*, and we have shown that VirR recognizes and binds to these alternative binding sites *in vitro* and activates reporter gene expression *in vivo* (10, 26).

In this article, we report the identification of VirR boxes upstream of the *netB* gene in the necrotic enteritis strain EHE-NE18, which led to the hypothesis that expression of *netB*, and hence the subsequent production of the toxin, is regulated by VirSR. Using *in vitro* binding assays and reporter gene expression assays, we show that these VirR boxes are functional. Mutation of the *virR* genes of EHE-NE18 and a second necrotic enteritis-causing strain, 56, reveals that the VirSR two-component signal transduction system is involved in the regulation of NetB production. Furthermore, quantitative real-time PCR (qRT-PCR) analysis of RNA isolated from EHE-NE18, its isogenic *virR* mutant, and a complemented derivative demonstrates that *netB* expression is regulated at the level of transcription. In summary, these results have revealed another toxin gene that is regulated by the VirSR system, thereby adding to the repertoire of genes in the ever-expanding VirSR regulon.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown at 37°C in 2× YT agar or broth or SOC broth (36) supplemented with chloramphenicol (30 μg/ml) or erythromycin (150 μg/ml). *C. perfringens* strains were cultured at 37°C in brain heart infusion broth (Oxoid), Trypticase-peptone-glucose (TPG) broth (35), fluid thioglycolate medium (FTG) (Difco), or nutrient agar supplemented with 30 μg/ml chloramphenicol ($NACm_{30}$), 7 μg/ml or 50 μg/ml erythromycin ($NAEm_7$ or $NAEm_{50}$, respectively), or 10 μg/ml rifampin and 10 μg/ml nalidixic acid ($NARif_{10}Nal_{10}$). For the screening of perfringolysin O (PFO) production, *C. perfringens* transformants were grown on horse blood agar (HBA) (22). All agar cultures of *C. perfringens* were incubated in an atmosphere of 10% (vol/vol) H_2 and 10% (vol/vol) CO_2 in N_2.

Molecular techniques. Plasmid DNA from *E. coli* cells was routinely isolated using an alkaline lysis method (25). When DNA was used for sequencing, an

TABLE 2. Oligonucleotides used in this work

Primer or use	Sequence (5′–3′)	Location or use[a]
Cloning of VirR boxes		
JRP4051	ACCAGTTATGTATAAATTTTGACCAGTTTTACCA	VirR boxes upstream of *netB* (+)
JRP4052	TGGTAAAACTGGTCAAAATTTATACATAACTGGT	VirR boxes upstream of *netB* (−)
Gel mobility shift analysis		
JRP589	GGAACTCATATTATAATTGG	Upstream of *pfoA* VirR boxes (+)
JRP618	CTCTAATTTTTTCTTTTCCC	Downstream of *pfoA* VirR boxes (+)
Construction of pJIR3566		
JRP2712	ATGATCAAGCTTGCATGCAAAAAATAAACTGAGAAAATGATATAC	*gdh* promoter (+)
JIR2713	ATGATCAAGCTTCATACCTAATTTATCACATGC	*gdh* promoter (−)
JRP4613	ACGCGTCGGAAACGTAAAAGAAGTTATGGAAATAAG	Construction of *ermB*-RAM cassette
JRP4614	GAACATAATGCTCATGTAATCACTCCTTCTTAATTAC	Construction of *ermB*-RAM cassette
JRP4615	GAGTGATTACATGAGCATTATGTTCAGTAAGGTCGTTAATC	Construction of *ermB*-RAM cassette
JRP4616	CTTGGGTTAATTGAGGCCTGAGTATAAGGTGACTTATAC	Construction of *ermB*-RAM cassette
JRP4617	CTCAGGCCTCAATTAACCCAAGAACAAAAATATAAAATATTCTC	Construction of *ermB*-RAM cassette
JRP4618	ACGCGTGGGGAATTATTTCCTCCCGTTAAATAATAG	Construction of *ermB*-RAM cassette
JRP4619	GAGTATTTATGAAAAGCGG	Replace native promoter of *ermB*-RAM with pasfdx promoter region
JRP4620	ATACAATAAGTTATGAGAAGGAGTGATTACATGAGCATTATG	Replace native promoter of *ermB*-RAM with pasfdx promoter region
JRP4621	GTCGACACGCGTGAAGATTTAGGATTTACTGTAAT	Replace native promoter of *ermB*-RAM with pasfdx promoter region
JRP4622	GTAATCACTCCTTCTCATAACTTATTGTATCATGTTTTTAAAC	Replace native promoter of *ermB*-RAM with pasfdx promoter region
JRP4042	AAAAAAGCTTCAGGAAACAGCTATGACCATGATTACGCCTAGCTTCCATGCCTGC	Amplification of *lacZ*α (+)
JRP4623	AAAAAATGTACAGGTGTTGGCGGGTGTCGGG	Amplification of *lacZ*α (−)
Targetron		
JRP4106	TGAACGCAAGTTTCTAATTTCGGTTTTAAGTCGATAGAGGAAAGTGTCT	EBS2 primer to retarget intron to *virR*
JRP4107	AAAAAAGCTTATAATTATCCTTACTTAACAATAAGGTGCGCCCAGATAGGGTG	IBS primer to retarget intron to *virR*
JRP4108	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAATAAGGGTAACTTACCTTTCTTTGT	EBS1 primer to retarget intron to *virR*
JRP3867	CGAAATTAGAAACTTGCGTTCAGTAAAC	Targetron universal primer
JRP4240	CGCGTCGGAAACGTAAAAGAAGTTATGGAAATAAG	Upstream of *ermB* RAM (+)
JRP4241	CTATTATTTAACGGGAGGAAATAATTCCCCACGCGT	Downstream of *ermB* RAM (−)
JRP182	GTTATGAAGTTCGTGCTTTTAG	Upstream of targetron insertion within *virR* (+); also used to generate *virR* probe
JRP2813	ACGCGTTCATTAACATATTAAATCCCC	Downstream of targetron insertion within *virR* (−)
JRP111	GTATTATTACCTTTCTCTCA	*virR* probe (−)
JRP2633	CCGGGATCCTTAGGGTAACAAAAAACACC	*catP* probe (−)
JRP2142	CTCAGTACTGAGAGGGAACTTAGATGGTAT	*catP* probe (+)
JRP3947	GGGAACGAAACGAAAGCG	Targetron probe (+)
JRP3948	CGTAATAAATATCTGGAC	Targetron probe (−)
JRP2369	AATAAGTAAACAGGTAACGTCT	*ermB* probe (+)
JRP2370	GCTCCTTGGAAGCTGTCAGTAG	*ermB* probe (−)
qRT-PCR		
JRP2479	CCATCTGTTTTTATATCTGCTCCAGTA	Within *rpoA* (+)
JRP2480	GGAAGGTGAAGGACCAAAAACTATT	Within *rpoA* (−)
JRP4707	AAATATACTTCTAGTGATACCGCTTCACA	Within *netB* (+)
JRP4708	GAGGATCTTCAATAAATGTTCCACTTAA	Within *netB* (−)

[a] +, sense primer; −, antisense primer.

additional polyethylene glycol precipitation step was included, as outlined in the instructions of the Prism Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Restriction endonucleases and other enzymes were used as specified by the manufacturer (Roche Diagnostics or New England Biolabs). All oligonucleotide primers are listed in Table 2.

Competent *E. coli* (17) and *C. perfringens* (37) cells were prepared and transformed as described previously unless otherwise indicated. *C. perfringens* genomic DNA was isolated from 5 ml FTG broth cultures as was done previously (28). PCR amplification was carried out as described previously (10). PCR products were either purified directly using the QIAquick PCR purification kit

(Qiagen) or extracted from agarose gels using the QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions.

Cloning of the *netB* VirR boxes into pJIR2373 was carried out as described previously (10). The resultant construct, pJIR3573, was used to transform the *C. perfringens pfoA* mutant, JIR4228 (Table 1), to yield JIR12345.

Construction of the vector used in targetron mutagenesis, pJIR3566. As part of another ongoing study, a new targetron vector was constructed for the mutagenesis experiments reported here. The vector, pJIR3566, utilizes a clostridial promoter and contains an *ermB* retrotransposition-activated marker (*ermB*-RAM). To construct pJIR3566, the P*gdh* promoter region from *Clostridium difficile* strain 630 was PCR amplified using primers JRP2712 and JRP2713. The resultant 207-bp fragment was then digested with HindIII and SphI and cloned into the targetron vector pJIR750ai (8), generating pJIR3753. The RP4 *oriT* region was PCR amplified from plasmid pJIR1456 (21), cloned into pCR2.1-TOPO (Invitrogen), digested with SphI and SpeI, and subcloned into the XbaI and SphI sites of pJIR3753, resulting in pJIR3754. The replication region of pMTL540F (15) was then excised using NheI and BstZ17I and subcloned into the FspI and SpeI sites of pJIR3754, leading to the construction of pJIR3464.

To facilitate one-step selection of chromosomal integrants, an *ermB* retrotransposition-activated marker (*ermB*-RAM was constructed by a series of stepwise splice overlap extension (SOE) PCRs (16) and cloning reactions that led to the insertion of the *td* group I intron and its minimal exon sequences (13) in reverse orientation within the *ermB* coding region, immediately downstream of the *ermB* start codon (Table 2). The native promoter of the *ermB*-RAM cassette was subsequently replaced with the strong *Clostridium pasteurianum* ferredoxin promoter (P*fd*) (pJIR3756) to maximize expression of *ermB*, and the modified cassette was cloned into the MluI site of pJIR3464 to give pJIR3562. Finally, to facilitate blue/white screening of retargeted targetron elements, the 5′ (retargeted) end of the group II intron within the resultant plasmid, pJIR3562, was replaced with the *lacZ*α gene, leading to the construction of the final clostridial targetron plasmid, pJIR3566.

Construction of the *virR* targetron plasmid. To identify potential targetron insertion sites, the nucleotide sequence of *virR* was submitted to the Sigma TargeTron design site (http://www.sigma-genosys.com/targetron/). Of the predicted sites, insertion into the sense strand at position 546/547 from the ATG start codon in *virR* was selected for targetron modification. To retarget the group II intron, primer-mediated mutation by PCR was carried out with the IBS, EBS2, EBS1/δ, and EBS universal primers (Table 2) in accordance with the instructions from the TargeTron gene knockout system (Sigma-Aldrich), with the exception that Phusion High-Fidelity DNA polymerase (NEB) and FailSafe buffer E (Epicentre Biotechnologies) were used in place of the JumpStart REDTaq Ready mix. The 350-bp gel-extracted retargeting PCR product was digested with HindIII and BsrGI and ligated into pJIR3566 DNA that had been digested with the same enzymes and alkaline phosphatase treated. The ligation mixture was used to transform *E. coli* TOP10 cells (Invitrogen), and plasmids from a selection of the resultant transformants were isolated and sequenced. A vector, pJIR3608, that contained the desired altered nucleotides, was chosen for subsequent mutagenesis studies.

Construction of *virR* mutants using the Targetron mutagenesis system. Approximately 5 μg of pJIR3608 was used to transform *C. perfringens* strains EHE-NE18 and 56 by electroporation. Transformed cells were inoculated into 20-ml brain heart infusion (BHI) broths and incubated overnight at 37°C. The resultant cultures were subsequently passaged twice, over a 24-h period, in 20 ml of BHI broth. Transformants containing potential intron insertions were selected on $NAEm_7$. Following overnight incubation at 37°C, selected colonies were patched onto $NACm_{30}$ and $NAEm_7$. Transformants that were resistant to erythromycin but sensitive to chloramphenicol were chosen for further analysis, since they represented derivatives that contained an inserted intron but were cured of pJIR3608. These transformants were also screened on HBA to assess the effect of the mutation on perfringolysin O production.

To show that the intron had inserted into the *virR* gene, genomic DNA was isolated as described previously (28) and analyzed by PCR with the primer pairs JRP2813/JRP182 or JRP4240/JRP4241. The former primer pair flanks the site of insertion within the *virR* gene, while the latter primer pair amplifies the *ermB*-RAM within the intron. To confirm the PCR results, Southern hybridization analysis was carried out. Briefly, genomic DNA of each strain was digested with BglII, separated by electrophoresis, transferred to a nylon membrane (GE Healthcare), and probed with PCR-amplified, digoxigenin (DIG)-labeled *virR*, *ermB*, *catP*, or intron-specific probes (Table 2). Blots were developed as per the manufacturer's instructions.

Perfringolysin O. *C. perfringens* strains were grown to late log phase before culture supernatants were collected by centrifugation as described previously (4). Perfringolysin O activity was determined by measuring the hemolysis of horse red blood cells in a doubling dilution assay, as described previously (42). The perfringolysin O titer was defined as the reciprocal of the last well that showed complete hemolysis, which was indicated by a significant decrease in absorbance at 570 nm. The PFO titers represent the means obtained from duplicate assays using supernatants derived from three independent cultures of each strain.

Gel mobility shift analysis. The 183-bp DNA target fragments (12) were PCR amplified with oligonucleotides JRP589 and JRP618 using the *pfoA* reporter constructs as templates. These DNA fragments subsequently were labeled with digoxigenin-11-ddUTP (Roche) as per the manufacturer's instructions. Gel mobility shift assays were performed with purified VirR protein as described previously (10, 12).

Isolation of RNA and qRT-PCR. EHE-NE18 cells were harvested at late logarithmic growth phase. Total RNA was isolated using the TriZol reagent (Invitrogen) as described previously (11). Conversion of 2 μg of RNA to cDNA and quantitative real time PCR (qRT-PCR) analysis were carried out as before (9) using *rpoA* or *netB* primers (Table 2). Total RNA or genomic DNA was isolated from at least three biological replicates from each strain and assayed in triplicate. The values obtained were normalized to that of the *rpoA* gene for each strain, and the results were expressed as a proportion of that for the wild type.

Western blot analysis. Two methods were used to analyze the production of NetB toxin. To determine when NetB toxin was produced, culture supernatants were harvested throughout the growth of EHE-NE18 in TPG broth. Each sample was separated by SDS-PAGE on a NuPAGE Novex 4 to 12% Bis-Tris gel (Invitrogen) in NuPAGE morpholineethanesulfonic acid (MES) SDS running buffer (Invitrogen). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (PALL) and probed with rabbit polyclonal rNetB antiserum (19). Blots were developed with the ECL Western blotting kit (Amersham Biosciences) as per the manufacturer's instructions.

To examine the production of NetB by EHE-NE18 and 56 *virR* mutants, culture supernatants were harvested at late logarithmic growth phase. The total protein concentration of the supernatants was determined using the bicinchoninic acid (BCA) protein assay kit. Approximately 200 μg of protein from each sample was separated by 12% SDS-PAGE. Proteins were then transferred and immobilized onto a nitrocellulose membrane (Millipore). The membrane was probed with polyclonal rabbit NetB antiserum (1:200) (19), followed by diluted (1:2,000) goat anti-rabbit antiserum secondary antibody (Millipore). Bound antibodies were detected using the Western Lightning Plus chemiluminescence reagent (Perkin Elmer) as per the manufacturer's instructions. Chemiluminescence was detected using X-ray film (Fujifilm).

Cell line and cytotoxicity assay. The chicken hepatoma cell line LMH (ATCC CRL-2117) was maintained in Earl's minimum essential medium (EMEM) supplemented with L-glutamine, 10% fetal calf serum (FCS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (100 μg/ml) (LMH cells also require 0.2% gelatin for adherence to surfaces) and incubated in a humidified environment of 5% CO_2 at 37°C.

To test for cytotoxicity, LMH cells were cultured to 70% confluence in 96-well plates (Nunc) grown in EMEM growth medium, with 5% FCS and without phenol red, at 37°C. *C. perfringens* strains were grown in TPG broth to a turbidity at 600 nm of 0.6. Culture supernatants were obtained by centrifugation at 18,000 × *g* for 10 min and then dialyzed in phosphate-buffered saline. Volume-normalized, dialyzed culture supernatants were added to the LMH cell medium and incubated for 4 h at 37°C. Lactate dehydrogenase (LDH) release into the supernatant was measured as an indicator of cytolysis using the Cyto-Tox (Promega) kit and expressed as a percentage of cytotoxicity.

RESULTS

VirR activates *pfoA* reporter gene expression by binding to *netB* VirR boxes. Sequence analysis of the *netB* promoter region of EHE-NE18 revealed the presence of potential VirR boxes upstream of the putative −35 box (Fig. 1A). The sequence and spatial organization of these potential VirR binding sites were similar to those of the VirR boxes located upstream of the *pfoA* (10), *vrr*, *virU*, *virT*, and *ccp* genes (31), all of which have been shown to be recognized by VirR (10) (Fig. 1B). Gel mobility shift experiments using the purified VirR protein and DIG-labeled target DNA demonstrated that VirR was able to recognize and bind to the *netB* VirR boxes *in vitro*. The two shifted bands, complex I (CI) and complex II (CII),

FIG. 1. Identification of VirR boxes. (A) Nucleotide sequence of the region upstream of the *netB* gene in strain EHE-NE18. The start of the *netB* coding sequence is indicated by the bent arrow, with the sequence in bold. The putative ribosome binding site is indicated by the asterisks, while the potential −10 and −35 boxes of the VirR-dependent promoter are labeled as −10 and −35, respectively and are underlined and shown in bold. The VirR boxes are in bold and boxed and are labeled VB1 and VB2. (B) Comparison of the sequence and spatial organization of the VirR boxes of the *pfoA*, *vrr*, *virU*, *virT*, *ccp*, and *netB* genes. The VirR boxes are in bold, boxed, and labeled VB1 and VB2. The *virRS*-dependent promoters are in bold, underlined, and denoted by −10 and −35. The promoters of *vrr*, *virU*, *virT*, and *ccp* were determined previously (31).

were similar to those observed with the control *pfoA* VirR box region (Fig. 2A).

To determine if the *netB* VirR boxes were functional, *pfoA* reporter plasmids were constructed as described previously (10) and used to transform the JIR325-derived *pfoA* mutant, JIR4228 (5). Perfringolysin O activity was used to measure the expression of the *pfoA* reporter gene. As expected, the results showed that the positive-control strain, which carried the *pfoA* VirR boxes cloned upstream of the reporter gene, produced levels of perfringolysin O activity that were similar to those of the wild type, while no activity was detected from the negative-control strain harboring the shuttle vector, pJIR751 (Fig. 2B). Background levels of perfringolysin O activity were observed from the strain carrying the pJIR2373 *pfoA* reporter gene vector, as previously observed (10). In contrast, a wild-type level of perfringolysin O activity was observed when the *netB* VirR boxes were cloned upstream of the reporter gene (Fig. 2B).

NetB is produced at high cell density. To determine when NetB is produced, Western blot analysis using polyclonal NetB antisera was carried out on culture supernatants that were isolated at various time points during the growth of strain EHE-NE18. The results showed that under these experimental conditions, NetB production begins 4 h after inoculation (logarithmic growth phase), with increasing production at later time points (Fig. 3).

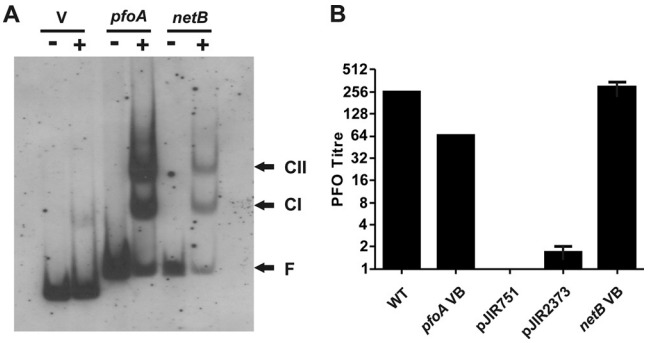

FIG. 2. Functional analysis of VirR boxes. (A) Gel mobility shift analysis of the VirR boxes upstream of *netB*. The DIG-labeled DNA targets containing the VirR boxes upstream of *pfoA* or *netB* were incubated in the absence (−) or presence (+) of 1 μg of purified VirR. The pJIR2373 vector control is denoted by V. The free DNA (F), CI, and CII bands are labeled and indicated by the arrows. (B) Perfringolysin O activity of reporter constructs. Perfringolysin O (PFO) titers of the JIR325 wild-type strain (WT), JIR4228 derivatives carrying reporter constructs containing the VirR boxes located upstream of the *pfoA* gene (*pfoA* VB) and the *netB* gene (*netB* VB), and JIR4228 derivatives with the shuttle vector (pJIR751) and the reporter gene vector (pJIR2373) are shown. The error bars represent the standard error of the mean; $n = 3$.

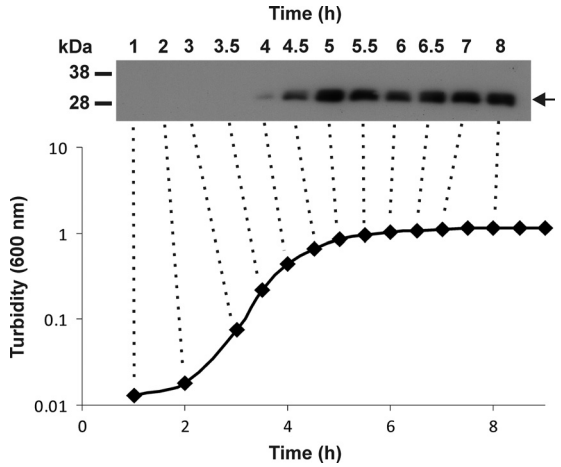

FIG. 3. Analysis of NetB toxin production in strain EHE-NE18. The growth of EHE-NE18 in TPG broth was monitored at 30-min intervals by measuring the turbidity at 600 nm. Culture supernatants were harvested at regular time points and analyzed by Western blotting using a polyclonal NetB antibody. The NetB protein is designated by the arrow, and the marker sizes are shown in kDa. The dotted lines link the selected culture supernatants to the corresponding time points.

Construction of *virR* mutants of EHE-NE18 and 56. Previous work showed that VirSR-regulated genes that have correctly spaced and positioned VirR boxes in the upstream promoter region are directly regulated by VirR (10, 12, 26, 31). To determine whether VirR regulated *netB* expression, the *virR* genes of two chicken necrotic enteritis-causing isolates, EHE-NE18 and 56, were insertionally inactivated by use of targetron technology (47). To aid in the construction of these mutants, we used a clostridial targetron vector, pJIR3566, which replicates in the clostridia, utilizes a strong clostridial promoter to drive targetron expression, and contains a retrotransposition-activated marker (47) that encodes erythromycin resistance to facilitate one-step selection of chromosomal integrants. Plasmid pJIR3566 also facilitates blue/white selection of retargeted introns due to the inclusion of the *lacZ*α gene fragment between the HindIII and BsrGI restriction sites, which are utilized during the intron "retargeting" procedure.

Of the predicted targetron sites, the insertion site within the region encoding the VirR DNA binding domain (FxRxHrS motif) was selected, since we previously demonstrated that mutation of this motif eliminated VirR function (23). The vector containing the *virR* targetron, pJIR3608, was introduced into EHE-NE18 and 56 by electroporation. As a result of the presence of the *ermB*-RAM, transformants containing potential insertions were selected on $NAEm_7$. Verification of targetron insertion into the desired site in the resultant mutants was obtained by PCR and Southern blotting (data not shown).

Mutation of the *virR* gene in the gas gangrene isolate strain 13 led to elimination of perfringolysin O production (38). Similar results were obtained with the EHE-NE18- and 56-derived *virR* mutants. When grown on HBA, the mutants showed no hemolytic activity (data not shown). This phenotype was confirmed by quantitative perfringolysin O assays. Compared to the wild-type strains, the *virR* mutants of EHE-NE18 and 56, JIR12405 and JIR12409, respectively, did not produce any detectable levels of perfringolysin O. Production of this toxin was restored to wild-type levels when the *virR* mutations were successfully complemented in *trans* by *virR* alone or by the wild-type *virRS* operon (Fig. 4A). The assay results confirmed that the targetron insertion had successfully inactivated VirR function.

VirR regulates NetB production in necrotic enteritis strains EHE-NE18 and 56. Culture supernatants were assayed to determine the effect of the *virR* mutations on NetB production. Western blot analysis using polyclonal NetB antisera demonstrated that NetB was being produced by both EHE-NE18 and 56. In contrast, no NetB was produced by the previously isolated EHE-NE18 *netB* mutant (Table 1) and the strain 13 derivative, JIR325. Very low, almost undetectable levels of NetB were observed with the *virR* mutants of both strains. The production of the NetB protein was restored when the mutation was complemented in *trans* with the wild-type *virR* genes or with the *virRS* operon (Fig. 4B). These results demonstrated that the production of NetB in these strains was positively regulated by the VirSR system.

To quantitate the level of NetB toxin activity, supernatants were analyzed in an LMH cell cytotoxicity assay, using lactate dehydrogenase (LDH) release from the cells as an indicator of cytolysis (19). The level of cytotoxicity observed with culture supernatants derived from the *virR* mutants was significantly reduced compared to the wild-type controls. In contrast, effectively wild-type levels of cytotoxicity were observed when supernatants from the complemented strains were tested (Fig. 5A). These results are consistent with those obtained in the Western blots and confirmed that NetB production was VirSR dependent.

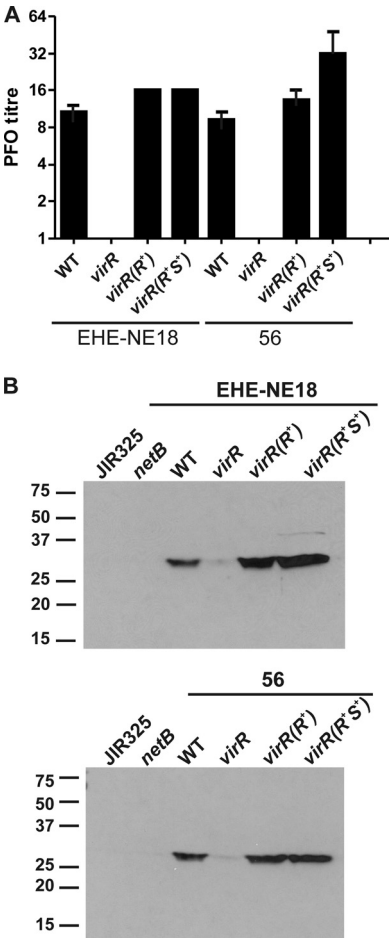

FIG. 4. Analysis of toxin production in isogenic derivatives. (A) Analysis of perfringolysin O production. The perfringolysin O (PFO) titers of the wild-type strains EHE-NE18 and 56 (WT), *virR* mutant derivatives carrying the pJIR750 vector (*virR*), and *virR* mutants complemented by *virR* [*virR*(R^+)] or the *virR*/*S* operon [*virR*(R^+S^+)] in *trans* on pJIR1897 and pJIR2529, respectively, are shown. The error bars represent the standard errors of the means (n = 3). (B) Western blot analysis of NetB production by *virR* mutants. Culture supernatants were isolated from JIR325, the EHE-NE18 *netB* mutant (*netB*), the wild-type strains (WT), *virR* mutants (*virR*), and *virR* mutants complemented with *virR* [*virR*(R^+)] or the *virRS* operon [*virR*(R^+S^+)]. Proteins from cell culture supernatants (200 μg) were separated by SDS-PAGE and subsequently transferred to nitrocellulose. The NetB protein was visualized by use of polyclonal NetB antisera followed by chemiluminescence detection. The wild-type strains from which the *virR* mutants were derived are indicated above each blot. The protein size markers (kDa) are indicated on the side of each blot.

To determine if regulation was mediated at the transcriptional level, the amount of *netB* transcript in EHE-NE18, its *virR* mutant, and the *virR* mutant complemented with the *virRS* operon was quantitated by use of qRT-PCR. The results

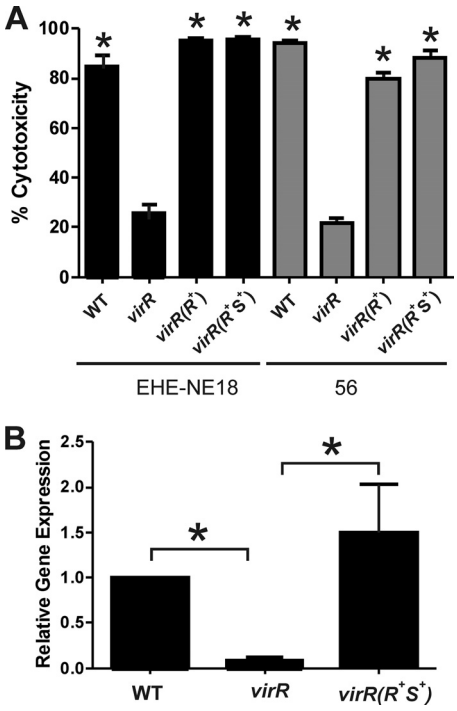

FIG. 5. Effect of virR mutation on NetB cytotoxicity and *netB* expression. (A) LMH cell cytotoxicity assay. Culture supernatants were isolated from JIR325, the wild-type strains (WT), *virR* mutants (*virR*), and *virR* mutants complemented with *virR* [*virR*(R^+)] or the *virRS* operon [*virR*(R^+S^+)]. The amount of cytotoxicity induced by NetB in each cell supernatant is expressed as a percentage and is indicated by the black bars (strain EHE-NE18) or gray bars (strain 56). The error bars represent the standard deviations, and the asterisks (*) denote a *P* value of <0.05 compared to results for the respective *virR* mutants, as calculated by nonparametric, two-tailed, Mann-Whitney *t* tests. (B) Quantitative real-time PCR analysis of *netB* transcription. Total RNA from EHE-NE18 (WT), the EHE-NE18 *virR* mutant (*virR*), and its complemented derivative [*virR*(R^+S^+)] was isolated from four biological replicates and assayed in triplicate. The expression levels of *netB* (black bars) in each strain relative to WT levels normalized to the *rpoA* gene (relative gene expression) are shown. The error bars represent the standard errors of the means, and the asterisks (*) represent a *P* value of <0.05 calculated by two-tailed Student's *t* test.

showed that *netB* transcription in the *virR* mutant was significantly reduced compared to that in the wild type but that expression was restored to wild-type levels upon complementation (Fig. 5B).

DISCUSSION

In this study, we identified potential VirR boxes upstream of the *netB* gene in the chicken necrotic enteritis-causing strain, EHE-NE18. Comparison of the VirR boxes found upstream of the *pfoA* gene with those associated with the *netB* gene revealed some sequence variation, but despite these differences, VirR was still able to recognize and bind to the *netB* VirR boxes *in vitro* and stimulate the expression of the *pfoA* reporter gene *in vivo*. Taken together, these results indicated that the VirR boxes upstream of *netB* were functional and suggested that *netB* transcription was regulated by the VirSR two-component signal transduction system. This hypothesis was confirmed by the construction of *virR* mutant derivatives of two necrotic enteritis strains, EHE-NE18 and 56, and their complementation by either *virR* or *virRS* in *trans*. Reduced NetB production was observed in the *virR* mutants and restored in the complemented derivatives. Further analysis showed that the low level of NetB produced in the *virR* mutant of EHE-NE18 was consistent with the significantly reduced level of *netB* transcript in comparison to those of the wild-type and complemented strains. These experiments provide clear evidence that NetB toxin production is regulated at the transcriptional level by the VirSR system.

These findings have significant implications for our understanding of the pathogenesis of avian necrotic enteritis infections. The VirSR system may function in a manner similar to that of the staphylococcal Agr quorum sensing system (27). Recent studies have indicated that in *C. perfringens* strain 13, the VirSR system is important in sensing an extracellular signal that leads to the transcriptional activation of toxin genes (30). In the staphylococcal Agr system, the extracellular signal is an autoinducing peptide that is a derivative of an AgrD propeptide that is modified by AgrB upon secretion (32). The Agr system represents a quorum sensing system that is responsive to cell density. We have identified similar *agrB* and *agrD* genes in the strain EHE-NE18 genome (data not shown), and other workers have proposed that in strain 13, the product of these genes is the autoinducing peptide that activates the VirSR system (30). Furthermore, in this study we have shown that NetB is not produced constitutively; it is produced only when the cells reach late logarithmic phase. Therefore, we postulate that VirSR-dependent NetB toxin production in *C. perfringens* is responsive to cell density.

Based on the data obtained in studies on strain 13 (30, 46), the results presented here, and our understanding of the pathogenesis of avian necrotic enteritis (45), we suggest that in the normal avian gastrointestinal tract, the low levels of *C. perfringens* cells are not sufficient to lead to induction of the VirSR system and significant NetB production. However, when the birds are suddenly changed to a high-energy, protein-rich diet, the growth of *C. perfringens* is stimulated. Therefore, its population density increases and the amount of autoinducing peptide secreted into the lumen of the gastrointestinal tract increases to the point where its concentration is high enough to lead to the induction of NetB toxin production by the induction of VirS, which subsequently activates the VirR response regulator. This mechanism would represent a highly efficient environmental adaption, since cytolytic toxins like NetB would be produced only when the *C. perfringens* population density was high and there was an increased probability of subsequent nutrient limitation. We propose that as with other *C. perfringens* toxins, the primary function of NetB is to increase nutrient availability by releasing complex macromolecules from cells of the host, not to cause any disease pathology.

To date, most studies on the VirSR system have been carried out on human gas gangrene or food poisoning strains of *C. perfringens*. Recently other workers showed that exposure of a *C. perfringens* type C strain to CaCo2 cells leads to VirSR-dependent upregulation of β-toxin and perfringolysin O production (46), and we have now shown that NetB toxin production in avian isolates of *C. perfringens* is VirSR regulated. Taken together, these studies demonstrate that the regulation of toxin production by the VirSR system is not limited to

human isolates but also extends to strains that cause disease in animals. Despite very significant differences in the diseases that these strains cause and the hosts that are affected, the method by which toxin production is regulated is essentially the same. These findings once more illustrate the global nature of this quorum-sensing VirSR two-component regulatory system and can again be interpreted on the basis that the major reason that *C. perfringens* strains produce specific hydrolytic and cytolytic toxins is to provide host-derived nutrients under conditions of high cell density.

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