The *Clostridium difficile* *cpr* Locus Is Regulated by a Noncontiguous Two-Component System in Response to Type A and B Lantibiotics

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The intestinal pathogen *Clostridium difficile* is known to grow only within the intestines of mammals, yet little is known about how the bacterium subsists in this environment. In the intestine, *C. difficile* must contend with innate defenses within the host, such as cationic antimicrobial peptides (CAMPs) produced by the host and the indigenous microbiota. In this study, we investigated the mechanism of activation and regulation of the CprABC transporter system, which provides resistance to multiple CAMPs and shows homology to the immunity systems of bacterial antimicrobial peptide producers. The CprABC system proved to be controlled by a noncontiguous two-component system consisting of the CprK sensor kinase and an orphan response regulator (CD3320; CprR). The CprK-CprR regulators were shown to activate *cprABCK* transcription in a manner similar to that by lantibiotic regulatory systems. Unlike lantibiotic producer regulation, regulation by CprK-CprR was activated by multiple lantibiotics produced by diverse Gram-positive bacteria. We identified a motif within these lantibiotics that is likely required for activation of *cpr*. Based on the similarities between the Cpr system and lantibiotic systems, we propose that the CprABC transporter and its regulators are relatives of lantibiotic systems that evolved to recognize multiple substrates to defend against toxins made by the intestinal microbiota.

Clostridium difficile is a Gram-positive spore-forming anaerobe that causes severe diarrheal disease. *C. difficile* enters the host as a dormant spore and germinates in the intestine, where it grows vegetatively and produces multiple toxins that contribute to disease symptoms (1–3). Many host risk factors that contribute to the probability of developing *C. difficile*-associated disease have been identified, but the bacterial factors that allow the bacterium to colonize the host to cause infections are unclear. Based on the known risk factors for contracting *C. difficile* infection (advanced age, antibiotic use, and hospitalization), the normal flora and the host immune system have vital roles in preventing *C. difficile* colonization and disease (4–6). The normal flora provides “colonization resistance,” which hinders *C. difficile* colonization. Loss of this resistance is typically precipitated by antimicrobial therapies (7).

Within the intestinal environment, *C. difficile* must defend itself against attacks by the host innate immune system and compete with intestinal microbiotas for nutrients and space. Both the host and the indigenous flora produce defensive molecules, such as cationic antimicrobial peptides (CAMPs), that keep intestinal invaders in check (8, 9). In previous work, we identified mechanisms that *C. difficile* uses to defend against cationic antimicrobial peptides (10, 11). One of these mechanisms, the *cpr* transporter system, was found to confer resistance to the CAMPs nisin and gallidermin, both of which are lantibiotics—a class of bacteriocins produced by many Gram-positive bacteria. The *cprABC* genes exhibit high sequence similarity to the lantibiotic immunity mechanisms found in lantibiotic producer strains. These immunity systems had not been described previously as providing resistance to these compounds outside the producer strains. Unlike typical lantibiotic immunity systems, the *cprABC* transporter confers resistance to multiple lantibiotic CAMPs and is not associated with lantibiotic biosynthesis genes or resistance to any endogenously produced lantibiotic toxin.

The *cpr* CAMP resistance mechanism is induced by exposure of cells to the lantibiotics nisin and gallidermin, but how this induction is mediated and regulated is not completely evident (11).

The *cpr* locus was identified through analysis of a mutant strain with increased resistance to CAMPs; the mutation was mapped to a gene encoding a putative orphan sensor histidine kinase (*cprK*) located immediately downstream from the *cpr* ABC transporter genes (*cprABC*). The *cprK* gene is transcribed concomitantly with the *cprABC* transporter genes, and overexpression of *cprK* alone activates transcription of the *cprABCK* operon, suggesting that CprK is a positive regulator of *cprABCK* transcription (11). CprK is not predicted to have DNA-binding domains and, therefore, would not likely be the sole regulator controlling *cpr* expression. However, no potential response regulator partner is located in the vicinity of the *cpr* operon.

In the current investigation, we identified a response regulator that responds to CprK and investigated lantibiotic recognition by CprK to determine regions of the lantibiotics that are required for activation of the system. We found that transcription of the *cprABCK* operon is controlled by a two-component regulatory system consisting of the CprK sensor kinase and an orphan response regulator encoded elsewhere on the chromosome. The sequences of the *cpr* regulators share similarity with lantibiotic regulatory proteins, and their mechanisms of function are indistinguishable from those of several characterized lantibiotic regulatory systems. We observed that unlike lantibiotic regulatory systems that control lantibiotic synthesis and immunity, the *cpr* regulators are activated by a variety of lantibiotics produced by a

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or features ^a	Source, construction, or reference ^b
Strains		
Lantibiotic producers		
<i>Actinoplanes garbadinensis</i> strain ATCC 31048	Actagardine	ATCC
<i>Bacillus subtilis</i> strain 168	Sublancin	58
<i>Bacillus subtilis</i> strain ATCC 6633	Subtilin	ATCC
<i>Enterococcus faecalis</i> strain DS16	Cytolysin	59
<i>Lactococcus lactis</i> strain ATCC 11454	Nisin A	ATCC
<i>Streptococcus mutans</i> strain ATCC 55676	Mutacin 1140	ATCC
<i>Streptomyces cinnamonensis</i> DSM 40005	Cinnamycin	DSM
<i>C. difficile</i>		
JIR8094	Erm ^s derivative of strain 630	60
MC119	JIR8094 <i>cprK1</i>	11
MC137	JIR8094 pMC123	11
MC141	JIR8094 <i>cprA::ermB</i>	11
MC146	JIR8094 MC141::Tn916 (<i>cprABC</i>); Erm ^r	11
MC167	JIR8094 pMC148	pMC148 → JIR8094
MC250	JIR8094 pMC205	pMC205 → JIR8094
MC251	JIR8094 pMC207	pMC207 → JIR8094
MC252	JIR8094 pMC206	pMC206 → JIR8094
<i>B. subtilis</i>		
BB3061	<i>trpC2 lacA::tet sacA::cat</i>	B. Belitsky
MC182	<i>trpC2 lacA::tet sacA::cat amyE::spc</i>	<i>amyE::spc</i> (DNA) → BB3061
MC184	<i>trpC2 lacA::tet sacA::cat amyE::Φ(cprAp::lacZ erm)</i>	pMC153 → MC182
MC186	<i>trpC2 lacA::tet sacA::Φ(cprR kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC154 → MC184
MC189	<i>trpC2 lacA::tet sacA::Φ(cprAp::cprKp::cprK kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC158 → MC184
MC190	<i>trpC2 lacA::tet sacA::Φ(cprR cprAp::cprKp::cprK kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC159 → MC184
MC191	<i>trpC2 lacA::tet sacA::Φ(cprR_{D51A} kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC163 → MC184
MC192	<i>trpC2 lacA::tet sacA::Φ(cprR_{D51E} kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC166 → MC184
MC193	<i>trpC2 lacA::tet sacA::Φ(cprAp::cprKp::cprK1 kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC160 → MC184
MC194	<i>trpC2 lacA::tet sacA::Φ(cprR cprAp::cprKp::cprK1 kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC162 → MC184
MC195	<i>trpC2 lacA::tet sacA::Φ(cprR_{D51A} cprAp::cprKp::cprK kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC164 → MC184
MC220	<i>trpC2 lacA::tet sacA::Φ(cprR cprAp::cprKp::cprK_{H250A} kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC177 → MC184
MC240	<i>trpC2 lacA::tet sacA::Φ(cprR cprAp::cprKp::spaK kan) amyE::Φ(cprAp::lacZ erm)</i>	pMC194 → MC184
<i>E. coli</i>		
DH5α	<i>supE44 ΔlacU169 (80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	61
Plasmids		
pCR2.1	<i>bla</i> , <i>kan</i>	Invitrogen
pMC123	<i>E. coli</i> - <i>C. difficile</i> shuttle vector; <i>bla</i> , <i>catP</i>	11
pHK23	<i>E. coli</i> plasmid for integration of <i>lacZ</i> transcriptional fusions at the <i>amyE</i> locus in <i>B. subtilis</i>	H. J. Kim (62)
pBB1364		63
pMC148	1.2 kb corresponding to CD3320 (<i>cprR</i>) and 500-bp upstream sequence cloned as BamHI/EcoRI into pMC123	oMC176/177
pMC153	336-bp <i>cprA</i> promoter fragment cloned as EcoRI/HindIII in pHK23 (<i>cprAp::lacZ</i>)	oMC215/216
pMC154	1.2-kb CD3320 (<i>cprR</i>) and upstream sequence cloned as KpnI/XbaI into pBB1364	oMC227/228
pMC158	2.2-kb fragment containing <i>cprAp</i> , 1.9-kb <i>cprK</i> and 426-bp upstream promoter sequence from <i>C. difficile</i> strain JIR8094 cloned as BamHI/EcoRI into pBB1364	oMC215/216 + oMC217/229
pMC159	2.2-kb <i>cprAp::cprKp::cprK</i> from pMC158 cloned as BamHI/EcoRI into pMC154	
pMC160	2.2-kb fragment containing <i>cprAp</i> , 1.9-kb <i>cprK1</i> and 426-bp upstream promoter sequence from <i>C. difficile</i> strain MC119 clones as BamHI/EcoRI into pBB1364	oMC215/216 + oMC217/229
pMC162	2.2-kb <i>cprAp::cprKp::cprK1</i> from pMC160 cloned as BamHI/EcoRI into pMC154	
pMC163	Same as pMC154 but <i>cprR_{D51A}</i>	oMC232/228 + oMC227/233
pMC164	1.2-kb <i>cprR_{D51A}</i> from pMC163 cloned as KpnI/XbaI into pMC158	
pMC166	Same as pMC154 but <i>cprR_{D51E}</i>	oMC234/228 + oMC227/235
pMC177	Same as pMC159 but <i>cprK_{H250A}</i>	oMC236/229 + oMC215/237
pMC194	Same as pMC159 but entire <i>spaK</i> coding sequence substituted for <i>cprK</i>	oMC215/294 + oMC292/293
pMC205	<i>cprAp::cprKp::cprK_{H250A}</i> from pMC177 cloned as BamHI/EcoRI into pMC123	
pMC206	1.2-kb <i>cprR_{D51A}</i> from pMC163 cloned as KpnI/XbaI into pMC123	
pMC207	1.2-kb <i>cprR_{D51E}</i> from pMC166 cloned as KpnI/XbaI into pMC123	

^a Erm^s, erythromycin susceptible; Erm^r, erythromycin resistant.^b Details of vector construction are available in File S2 in the supplemental material.

diverse assortment of Gram-positive organisms. Based on these observations, we conclude that the Cpr ABC transporter and its regulators are replicas of lantibiotic immunity systems that evolved to recognize multiple ligands and defend *C. difficile* against toxins made by other bacteria in the intestine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Clostridium difficile* strains were routinely cultured in BHIS medium (12) with or without supplementation with 0.1% L-cysteine. Media for growth of *C. difficile* were supple-

mented with 10 μg thiamphenicol ml^{-1} or 5 μg erythromycin ml^{-1} , 10 μg cinnamycin ml^{-1} (Sigma-Aldrich), or nisin (MP Biomedicals) as necessary. *C. difficile* strains were maintained at 37°C in an anaerobic chamber (Coy Laboratory Products) with an atmosphere of 10% H_2 , 5% CO_2 , and 85% N_2 as previously described (13). *Streptococcus mutans* was cultured on brain heart infusion (BHI) broth or nutrient agar at 37°C in an atmosphere containing 5% CO_2 . *Enterococcus faecalis* and *Lactococcus lactis* were cultured in BHI or tryptone yeast extract (TY) medium at 37°C without aeration. *Streptomyces cinnamomeus* and *Actinoplanes garbadi-nensis* were grown at 30°C on glucose yeast malt (GYM) medium and nutrient agar, respectively. *Escherichia coli* strains were grown at 37°C in L medium (14) or BHIS medium supplemented with 20 μg chloramphenicol ml^{-1} or 100 μg ampicillin ml^{-1} as needed. *Bacillus subtilis* strains were routinely grown at 37°C in L broth or TY medium (15) supplemented with 1 μg erythromycin ml^{-1} , 50 μg spectinomycin ml^{-1} , 2.5 μg neomycin ml^{-1} , or 2.5 to 5 μg chloramphenicol ml^{-1} when needed.

Strain and plasmid construction. Oligonucleotides used in this study are listed in File S1 in the supplemental material. Genomic DNAs used for PCR amplification include *C. difficile* strain 630 DNA (GenBank accession number AM180355) (16, 17), *Bacillus subtilis* strain 168 (GenBank accession number AL009126) (18), and *Bacillus subtilis* strain ATCC 6633 (GenBank accession number U09819) (19, 20). Details of DNA cloning and vector construction are outlined in File S2 in the supplemental material. Conjugation of plasmids from *E. coli* into *C. difficile* was performed as previously described (10). Isolation of genomic and plasmid DNA for transformation of *B. subtilis* and *E. coli* was performed by following standard protocols. Transformation of competent *B. subtilis* cells was carried out by the method of Yasbin et al. (21). Cloned DNA fragments were verified by sequencing through the Tufts University Core Facility using an ABI 3130XL DNA sequencer.

Quantitative reverse transcription-PCR (qRT-PCR) analysis. Log-phase cultures of *C. difficile* grown in BHIS medium or BHIS supplemented with nisin, cinnamycin, or subtilin supernatant were harvested (at an optical density at 600 nm [OD₆₀₀] of 0.4 for nisin samples and approximately 0.7 for cinnamycin and subtilin samples), and RNA was prepared as previously described (11, 22). As an additional control, non-subtilin-producing supernatants of *B. subtilis* were tested for potential effects on *C. difficile*, which were negative. RNA quantification and quality were measured by absorbance (A_{260} and A_{260}/A_{280} ratio, respectively) on a Nano-Drop ND-1000 spectrophotometer (Thermo Scientific). Primers for quantitative PCR (qPCR) were designed using the online PrimerQuest tool by IDT, found at <http://www.idtdna.com/Scitools/Applications/Primerquest>, and amplification efficiencies for each primer set were determined prior to use. cDNA synthesis was performed with random hexamers using the Tetro cDNA synthesis kit (Bioline). Mock cDNA synthesis reaction mixtures containing no reverse transcriptase were performed to control for chromosomal DNA contamination in subsequent amplifications. cDNA samples were diluted 4-fold and used as templates for quantitative real-time PCR of *rpoC* (primers oMC44 and oMC45), *cprA* (primers oMC96 and oMC97), and CD3320 (primers oMC174 and oMC175) using a SensiMix SYBR and fluoresein kit (Bioline) and a Bio-Rad CFX96 real-time system. Reactions were performed using 4 μl of diluted cDNA and 1 μM each primer in a final volume of 20 μl . Technical triplicate reactions were performed using cDNA extracted from each of a minimum of three biological replicates, and results are presented as the means and standard deviations of the data obtained. Amplification included 40 cycles of the following steps: 15 s at 95°C, 15 s at 53°C, and 15 s at 72°C. Results were calculated using the comparative cycle threshold method (23), in which the amount of target mRNA is normalized relative to an internal control transcript (*rpoC*). The two-tailed Student *t* test was used to analyze the data.

β -Galactosidase assays. *B. subtilis* strains carrying the *Pcpr::lacZ* transcriptional fusion at the *amyE* locus and combinations of wild-type or mutated *cprR* and *cprK* alleles integrated at *sacA* were grown in TY medium or TY medium supplemented with 5 μg nisin ml^{-1} , 10% subtilin

culture filtrate, or mock culture filtrate. The subtilin producer strain, *B. subtilis* ATCC 6633, was inoculated into TY medium (pH 7.4) and grown at 37°C with vigorous shaking. Subtilin-producing cultures were centrifuged to pellet the cells, and supernatants were filtered through a 0.2- μM filter to remove bacteria before addition to fresh TY medium (pH 7.4) for β -galactosidase experiments. As a control, the *B. subtilis* *spa*-negative strain BB3061 was grown and the filtered supernatant was tested for possible effects of the spent medium on reporter expression (none detected). Cells were grown to an OD₆₀₀ of approximately 1.0, harvested, and frozen at -20°C until assayed. β -Galactosidase assays were performed as previously described (24), and the specific activity was determined as described by Miller (25). Each experiment included a minimum of 3 biological replicates, and the results are presented as the means and standard deviations of the data obtained. The two-tailed Student *t* test was used to analyze the data.

RESULTS

Identification of a candidate response regulator for regulation of the *cpr* operon.

In a previous study, we found that the *cprK* gain-of-function mutant (MC119; *cprK1*) had greatly increased expression of the *cprABCK* genes, which resulted in much higher resistance to lantibiotic CAMPs than with the wild-type strain (11). These results led us to conclude that the CprK histidine kinase (HK) is a positive regulator of the *cpr* operon. Though CprK is involved in regulation of the *cpr* operon, this protein does not have any predicted DNA-binding domains for directly influencing transcriptional regulation. The simplest explanation for how CprK regulates transcription of *cprABCK* is through interaction with a second regulatory protein that has DNA-binding capabilities, that is, as part of a two-component regulatory system (TCS) (26). Because no response regulators are encoded near the *cprABCK* operon, we hypothesized that one or more cognate DNA-binding response regulators (RR) that can be activated by CprK were located elsewhere on the chromosome. *C. difficile* has 51 annotated response regulators: 45 appear to be part of two-component system operons, and 6 are considered orphan response regulators. To narrow down the list of potential response regulators that may be involved in *cpr* regulation, we searched available genome sequences for homologs of the CprABCK proteins in all sequenced *C. difficile* strains as well as for orthologs in other Firmicutes, including *Bacillus anthracis*, *Bacillus cereus*, and *Paenibacillus* sp. In several cases, we identified ABC transporter proteins with high similarity to the CprABC ABC transporter, most of which were closely linked to putative histidine kinase and response regulator genes. In addition, the genes in the *cprABCK* operon share significant homology to the lantibiotic immunity mechanisms found in lantibiotic-producing bacteria, many of which are regulated by two-component regulatory systems consisting of a membrane-spanning sensor histidine kinase and a DNA-binding response regulator. Of the characterized lantibiotic systems, the *cpr* operon most closely resembles the immunity and regulatory regions of the subtilin system (Fig. 1). Based on these clues, we performed a BLAST search of the *C. difficile* genome for homologues of the SpaR lantibiotic response regulator and response regulators similar to those identified near orthologues of the *cprABCK* genes in other species. In each case, the searches revealed the same *C. difficile* putative open reading frame as having the highest similarity to the response regulators queried. This gene, CD3320, encodes a putative response regulator that is conserved in all of the *C. difficile* genomes for which sequence data are available. *cprK* and CD3320 are considerably distant from each

FIG 1 The *C. difficile* *cpr* operon (*cprABCK*) and orphan response regulator CD3320 share homology to the *spa* immunity and regulatory proteins of the lantibiotic producer *B. subtilis* ATCC 6633. Genetic organization of the *C. difficile* strain 630 *cpr* regions (CD1348 lipoprotein [LP], *cprABC* ABC transporter, ABC-binding cassettes and permeases, and the orphan histidine kinase *cprK*) compared with the *spaIFEGRK* immunity and regulatory genes of the subtilin biosynthetic locus. Numbers indicate percent similarity of proteins to the corresponding *C. difficile* sequences.

other on the chromosome (1.58 Mb and 3.88 Mb from the origin of replication, respectively). Based on the high similarity to lantibiotic response regulators, we predicted that CD3320 was the most likely candidate response regulator for CprK.

Effects of CAMP exposure on CD3320 expression and the influence of CD3320 expression on *C. difficile* growth in CAMPs. *cprK* is not cotranscribed with its partner response regulator, and the potential interactions between these factors are unknown; therefore, we were not able to infer whether the cognate response regulator would be a positive or a negative regulator of *cpr* expression. To investigate the potential role of CD3320 in the regulation of the *cpr* operon, we introduced a plasmid-based copy of the CD3320 gene and its upstream region (pMC148) into wild-type *C. difficile* strain 630E via conjugation. *C. difficile* carrying the parent vector, pMC123, was used as a control. Wild-type strains carrying pMC123 (MC137) or pMC148 (MC167) were grown in BHIS medium supplemented with 10 μg thiamphenicol ml^{-1} for maintenance of the plasmid, with or without the addition of 10 μg nisin ml^{-1} (Fig. 2). Both strains grew at the same rate in the absence of nisin, but *C. difficile* carrying the plasmid with CD3320 was able to adapt to nisin about 2 h sooner than the wild type carrying the vector alone. To confirm that increased CD3320 expression altered transcription of the *cpr* operon and led to faster adaptation to nisin, we performed real-time quantitative PCR

analyses for the CD3320 and *cprA* transcripts. Addition of a plasmid-based copy of CD3320 to wild-type cells (MC167) resulted in an average of 6-fold-higher transcript levels for CD3320 in *C. difficile* grown in BHIS broth (Fig. 3). Expression of CD3320 in *C. difficile* carrying the empty vector only (MC137) did not increase when the cells were exposed to nisin (data not shown). As anticipated, bacteria with the plasmid-based CD3320 response regulator had increased expression of the *cpr* operon, even without the addition of nisin to the growth medium (Fig. 3). These data suggest that higher basal-level expression of CD3320 allows cells to adapt faster to CAMPs but does not result in a significant increase in *cpr* expression when CAMPs are present. Taken together, these results indicate that CD3320 is a positive regulator of *cprABCK* transcription and suggest that additional factors may influence transcriptional activation by the CD3320 response regulator.

Dissection of *cpr* regulation in a heterologous system. Although overproduction of CD3320 in *C. difficile* resulted in faster adaptation to the CAMP nisin and augmented the expression of the *cpr* operon, we considered the possibility that these may be indirect effects mediated through additional factors. To resolve whether CD3320 was directly or indirectly affecting transcription of the *cpr* operon, we looked for more direct evidence of the effects of the CD3320 response regulator on *cpr* expression. Initially, we sought to create an insertional disruption in the coding sequence

FIG 2 Effects of the CD3320 orphan response regulator CprR on growth in the lantibiotic CAMP nisin. Wild-type *C. difficile* strain 630E carrying control vector pMC123 (MC137; circles), pMC123::cprR (MC167; triangles), pMC123::cprR_{D51E} (MC251; squares), pMC123::cprR_{D51A} (MC252; diamonds), or pMC123::cprK_{H250A} (MC250; exes) was grown in BHIS medium with or without the addition of 10 μg nisin ml^{-1} . The growth rates of strains in BHIS alone were indistinguishable and are thus shown as a single gray line for clarity.

FIG 3 qRT-PCR analysis of *cprR* (CD3320) and *cprA* expression with the addition of plasmid-encoded *cprR*, *cprR_{D51A}*, *cprR_{D51E}* or *cprK_{H250A}*. *C. difficile* wild-type strain 630E carrying the control vector pMC123 (MC137), pMC123::*cprR* (MC167), pMC123::*cprR_{D51A}* (MC252), pMC123::*cprR_{D51E}* (MC251), or pMC123::*cprK_{H250A}* (MC250) was grown in BHIS medium to an OD₆₀₀ of 0.4. RNA was harvested, cDNA was synthesized, and qPCR was performed using gene-specific primers for CD3320 (A) or *cprA* (B). Results were normalized to an internal control gene (*rpoC*) and graphed as the ratio of each transcript to that of *rpoC* and then normalized to the wild-type vector-only control grown in BHIS. The means and standard deviations of a minimum of three biological replicates are shown (*, $P \leq 0.05$ by a Student two-tailed *t* test).

of CD3320 using a TargeTron-based group II intron (27), but we were unable to disrupt CD3320 by this method. In lieu of an insertional disruption, we recapitulated the putative *cpr* regulatory factors in the heterologous host *Bacillus subtilis*. A series of constructs were created to test the individual and combined effects of the regulatory components on transcription from the *cpr* promoter, as described in File S2 in the supplemental material. To measure transcription, a fragment containing the *cpr* promoter was fused to the *lacZ* reporter gene (*cprAp::lacZ*; pMC153) and integrated into the *B. subtilis* chromosome at the nonessential *amyE* locus (MC184). Additional constructs containing the genes and promoter regions for *cprK* (pMC158), CD3320 (pMC154), or both (pMC159) were integrated at the *sacA* locus of MC184 to create strains MC189, MC186, and MC190, respectively.

B. subtilis strains containing the various regulatory components were then grown in rich medium (TY) with or without nisin, and samples were taken for determination of β -galactosidase activity, the results of which are listed in Table 2. In a strain carrying only the

cprAp::lacZ fusion (MC184), β -galactosidase activity was consistently low with or without nisin added to the medium, indicating that transcription from the *cpr* promoter is not highly activated by endogenous factors present in the *B. subtilis* host. We also observed low β -galactosidase activity with or without nisin for the strain carrying the *cprAp::lacZ* reporter and *cprK* (MC189), affirming our hypothesis that CprK alone does not regulate transcription of the *cpr* genes. In contrast, a strain carrying the reporter fusion and the CD3320 response regulator (MC186) exhibited constitutively high β -galactosidase activity regardless of whether nisin was present in the growth medium. This result confirms that the CD3320 regulator can affect transcription from the *cpr* promoter and that the protein has a direct, positive influence on transcription. Moreover, in the strain containing the *cprAp::lacZ* reporter, CD3320, and *cprK* (MC190), we observed low β -galactosidase activity during growth in rich medium alone and markedly increased activity in the presence of either nisin or subtilin (Table 2; see also Table S3 in the

TABLE 2 β -Galactosidase activity of *B. subtilis* strains expressing *cpr* genes and variants^a

Strain	Relevant genotype	Activity in:			
		BHIS		BHIS + nisin	
		Mean $\pm$ SD	Ratio vs WT ^b	Mean $\pm$ SD	Ratio vs WT ^b
MC184	<i>cprAp::lacZ</i>	2.4 $\pm$ 0.4	0.3	3.1 $\pm$ 0.4	<0.1
MC186	<i>cprAp::lacZ</i> CD3320 (<i>cprR</i>)	86.4 $\pm$ 23.3	11.7	108.8 $\pm$ 16	0.4
MC189	<i>cprAp::lacZ</i> <i>cprK</i>	3.1 $\pm$ 0.8	0.4	2.9 $\pm$ 1.4	<0.1
MC190	<i>cprAp::lacZ</i> <i>cprR</i> <i>cprK</i>	7.5 $\pm$ 2.5	1.0	261.3 $\pm$ 24.6	1.0
MC193	<i>cprAp::lacZ</i> <i>cprK1</i>	3.3 $\pm$ 1.2	0.4	4.8 $\pm$ 1.3	<0.1
MC194	<i>cprAp::lacZ</i> <i>cprR</i> <i>cprK1</i>	195.3 $\pm$ 25.0	26.5	865.9 $\pm$ 199	3.3
MC191	<i>cprAp::lacZ</i> <i>cprR_{D51A}</i>	7.4 $\pm$ 2.4	1.0	8.3 $\pm$ 1.0	<0.1
MC192	<i>cprAp::lacZ</i> <i>cprR_{D51E}</i>	73.7 $\pm$ 8.3	10.0	76.6 $\pm$ 4.3	0.3
MC195	<i>cprAp::lacZ</i> <i>cprR_{D51A}</i> <i>cprK</i>	3.6 $\pm$ 2.7	0.5	4.1 $\pm$ 2.7	<0.1
MC220	<i>cprAp::lacZ</i> <i>cprR</i> <i>cprK_{H250A}</i>	2.1 $\pm$ 1.8	0.3	2.4 $\pm$ 1.5	<0.1
MC240	<i>cprAp::lacZ</i> <i>cprR</i> <i>spaK</i>	85.4 $\pm$ 17.6	11.6	73.2 $\pm$ 21.1	0.3

^a β -Galactosidase activity (Miller units) is shown as the mean and standard deviation (SD) from a minimum of 3 biological replicates. Bold type indicates a statistically significant difference in values obtained from growth in nisin compared to growth medium alone ($P \leq 0.05$ by a Student two-tailed *t* test).

^b The ratio represents the expression value for each strain compared to strain MC190 expressing wild-type CD3320 (*cprR*) and *cprK* in each medium.

supplemental material). In fact, the β -galactosidase activity in MC190 exposed to nisin was more than double that observed in the strain that had CD3320 without *cprK* (MC186) and was similar to the nisin-inducible expression observed in *C. difficile* (28). We also examined the activity of a strain harboring the reporter fusion, *cprR*, and a mutated allele of *cprK*, *cprK1*, from the hyperresistant *C. difficile* mutant MC119 (11). Consistent with the high level of *cprABCK* expression observed in *C. difficile* for the MC119 mutant, we observed exceptionally high β -galactosidase activity in *B. subtilis* carrying the *cprK1* mutation. Taken together, these data demonstrate that CD3320 is a response regulator that responds to nisin and subtilin via CprK and that CprK negatively regulates CD3320 during growth without CAMPs and is a positive regulator of CD3320 in the presence of CAMPs. In view of these results, we propose the designation *cprR* for the CD3320 orphan response regulator.

Regulation of CprR activity by CprK is likely mediated by phosphorylation. Though the data indicate that CprK and CprR can act together to activate transcription of the *cpr* operon, the results do not explain how these proteins interact to achieve this activation. As CprK and CprR appear to encode the sensor histidine kinase and response regulator of a two-component regulatory system, we presumed that activation and/or inactivation of the response regulator would occur through phosphorylation by the sensor kinase. Because transcription from *cprAp* occurs only in the presence of *cprR*, we infer that CprR binds to the *cpr* promoter to activate transcription. But the phosphorylation state of active CprR is not known. Based on the aforementioned results, one of two regulatory models is possible: (i) CprR is active in the unphosphorylated state or (ii) CprR is active in the phosphorylated state. In model i, CprK transfers phosphate to CprR under noninducing conditions and facilitates the loss of phosphate in the presence of CAMPs. In model ii, CprR is phosphorylated by CprK under inducing conditions (or by an additional factor in *B. subtilis*) and dephosphorylated under noninducing conditions.

To determine whether CprR is activated or inactivated by phosphorylation, we identified by sequence homology the aspartate residue (D51) that is the most likely site of phosphorylation of CprR and the histidine residue of CprK (H250) that is the most likely site of autophosphorylation and introduced site-directed mutations in these residues to analyze their roles in transcriptional activation. We created mutated versions of CprR in which D51 was changed to either alanine or glutamate (*cprR_{D51A}* and *cprR_{D51E}*). Alanine or glutamate substitutions at the phospho-accepting aspartate residue are expected to inhibit or mimic phosphorylation of response regulators, respectively (29). The conserved histidine residue of CprK was changed to an alanine to inhibit the kinase activity of the sensor (*cprK_{H250A}*). As shown in Table 2, strains expressing *cprR_{D51A}* (MC191) or both *cprR_{D51A}* and *cprK* (MC195) failed to express the *cprAp::lacZ* fusion, regardless of the presence of nisin. Conversely, a strain carrying *cprR_{D51E}* (MC192) had constitutively high levels of β -galactosidase activity, comparable to that of a strain expressing wild-type *cprR* (MC186). Moreover, a strain expressing both *cprR* and *cprK_{H250A}* (MC220) demonstrated very low β -galactosidase activity with or without nisin.

Finally, because CprK and the SpaK sensor (involved in subtilin regulation) have high sequence similarity and are structurally analogous, we tested the ability of a wild-type SpaK sensor to activate the *cprA* promoter (MC240). We found that SpaK negatively regulated *cprAp::lacZ* expression in the presence of subtilin

but was not affected by nisin (Table 2; see also Table S3 in the supplemental material). Thus, these results are consistent with the model that CprR activates transcription upon phosphorylation and is inactivated by dephosphorylation. Specifically, these data indicate that both phosphorylation and dephosphorylation of CprR can be modulated through CprK, though in *B. subtilis*, phosphorylation of CprR may also occur through an endogenous process (e.g., aspartyl phosphate or another histidine kinase). Furthermore, the inactivation of CprR by the *cprK_{H250A}* mutant shows that the phosphorylation and dephosphorylation mediated through CprK are independent processes.

Although we were unable to test null mutants of CprR and CprK, we were able to measure the effects of CprR and CprK phospho-domain mutations on *cpr* expression in *C. difficile*. We introduced plasmid-based copies of the *cprR_{D51A}*, *cprR_{D51E}*, and *cprK_{H250A}* mutant alleles into wild-type *C. difficile* to create merodiploid strains expressing both wild-type and mutated versions of each allele. Merodiploids were used to determine whether a mutant allele was dominant over the wild-type version of each protein, thereby indicating their relationship to the output phenotype. As shown in Fig. 2, the growth of strains expressing a defective *cprR_{D51A}* or *cprK_{H250A}* demonstrated delayed adaptation to nisin, while the strain expressing *cprR_{D51E}* adapted faster than the wild type. Although the *cprR_{D51A}* merodiploid has a growth delay in nisin, the wild-type copy of CprR in this strain eventually activates expression of the *cpr* genes (Fig. 3). All strains eventually reached the same optical densities as the wild type when grown in the presence of nisin (data not shown). The growth phenotypes of these merodiploid strains are supported by their expression profiles for *cprR* and *cprA* prior to nisin induction (Fig. 3). We observed an increase in *cprA* expression in the strain carrying the *cprR_{D51E}* (MC251) allele on a plasmid and decreased *cprA* expression in the strain containing *cprK_{H250A}* (MC250). Growth of the *cprK_{H250A}* merodiploid strain in nisin mirrors the defect in adaptation found in a *cprA* null mutant (Fig. 2) (11). This phenotype is likely because the *cprK_{H250A}* allele retains the ability to dephosphorylate the response regulator, leading to less activated response regulator and, consequently, less *cprABCK* expression. Together, the results observed in *B. subtilis* and *C. difficile* demonstrate that CprR is the downstream effector of *cpr* activation and that wild-type CprR cannot properly regulate transcription without a functional CprK.

The CprK-CprR two-component system is activated in response to type A and type B lantibiotic CAMPs. As mentioned above, the *cprABCK* operon and *cprR* gene have substantial homology to the immunity and regulatory genes found in lantibiotic production systems. The lantibiotic immunity and regulatory systems are highly specific for the lantibiotics produced by each system, and cross-immunity or -activation of these systems is very rare and of limited range (30–32). Unlike conventional lantibiotic systems, the *cprABCK-cprR* immunity and regulatory system is turned on by and provides resistance to at least two distinct lantibiotics: nisin and gallidermin (11). In an effort to understand how the CprK-CprR regulatory system is activated by multiple substrates, we investigated which lantibiotics are capable of activating transcription from the *cpr* promoter. Lantibiotics representing the two most common subclasses, the linear type A and globular type B peptides (33, 34), were assayed. A lawn of the *B. subtilis* indicator strain, MC190 (*cprAp::lacZ cprK⁺ cprR⁺*), was plated on L agar containing the colorimetric substrate X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), and paper disks containing subcultures of strains producing type A lantibi-

FIG 4 The CprK-CprR two-component system regulates expression from *P_{cpr}* in response to type A and type B lantibiotic CAMPs. Lantibiotics representing the two most common subclasses, the linear type A and globular type B peptides, were assayed for their ability to activate the CprK-CprR two-component system. A lawn of the *B. subtilis* indicator strain (*cprAp::lacZ cprK cprR*) was plated onto L agar containing the colorimetric substrate X-Gal. Purified peptide (2.5 μ g gallidermin [A]) or producer (nisin, *Lactococcus lactis* ATCC 11454 [B]; subtilin, *B. subtilis* ATCC 6633 [C]; mutacin 1140, *Streptococcus mutans* JH1140 [D]; cytolysin, *Enterococcus faecalis* DS16 [E]; sublancin, *B. subtilis* 168 [F]; actagardine, *A. linguriae* ATCC 31048 [G]; cinnamycin, *S. cinnamomeus* DSM40005 [H]) strains were impregnated on paper disks or placed directly on plates and incubated at 30°C. Lantibiotic production for each compound was verified by inhibition of *B. subtilis* growth on agar medium. Type A, nisin, gallidermin, subtilin, sublancin, cytolysin, and mutacin 1140; type B, cinnamycin and actagardine. Blue color indicates transcription from the *cprAp::lacZ* promoter fusion.

otics (nisin, gallidermin, subtilin, and mutacin 1140), type B lantibiotics (actagardine and cinnamycin), or the lantibiotic-like compound sublancin (35) were placed at different locations on the agar surface. Plates were incubated as indicated in Materials and Methods and assessed for blue color, signifying transcription from the *cprAp::lacZ* reporter fusion. As demonstrated in Fig. 4, cultures producing the lantibiotics nisin, gallidermin, subtilin, mutacin 1140, and cinnamycin activated the CprK-CprR regulators, resulting in β -galactosidase activity, while sublancin, the two-component cytolysin, and actagardine did not. Previously, we demonstrated that nisin and gallidermin both affect expression of the *cpr* operon and that the CprABC transporter provides resistance to these compounds. Based on the lantibiotic activation of the *cpr* promoter in *B. subtilis*, we tested the effects of some of the additional lantibiotic compounds on the activation of *cpr* expres-

sion in *C. difficile*. *C. difficile* strains were grown in the presence of 10 μ g cinnamycin ml^{-1} or supernatants containing subtilin (15% [vol/vol]) and analyzed by qPCR for expression of *cprA* (Fig. 5). Both cinnamycin and subtilin caused an increase in the expression of *cprA* in *C. difficile*. Thus, the CprK-CprR regulatory system can be activated by many lantibiotics, and this activation is not specific to either the type A or type B structural subclasses. But activation of *cpr* is not universal for all lantibiotics.

DISCUSSION

Resistance to CAMPs is an established virulence property for many bacterial pathogens (8, 9, 36, 37). In the context of human pathogens, however, bacterial CAMP resistance has been thought of primarily as a bacterial retort to specific antimicrobial peptide defenses encoded by the host genome. That is, bacteria evolved CAMP resistance mech-

FIG 5 qRT-PCR analysis of *cprA* expression during growth in cinnamycin and subtilin. *C. difficile* wild type (JIR8094) was grown in BHIS alone or BHIS supplemented with 10 μ g/ml cinnamycin or 15% supernatant from the subtilin producer strain, ATCC 6633, as described in Materials and Methods. Cells were harvested, RNA was extracted, cDNA was synthesized, and qPCR was performed using gene-specific primers for *cprA*. Results were normalized to an internal control gene (*rpoC*) and graphed as the ratio of each transcript level relative to that of the wild-type strain grown without supplementation. The means and standard deviations of three biological replicates are shown.

FIG 6 Model for *cpr*-mediated multiple lantibiotic resistance. The CprK, CprR, and CprABC proteins share significant homology to the immunity and regulatory systems of lantibiotic-producing bacteria. But, unlike typical producer-immunity mechanisms, the Cpr system confers resistance to a broad range of lantibiotic substrates. Lantibiotic substrates bind to CprK, resulting in activation and autophosphorylation of CprK. Upon activation, CprK transfers a phosphate group to the CprR response regulator, which allows the regulator to bind the *cpr* promoter and initiate transcription of the *cprABC* operon. The CprABC ABC transporter system can then bind to and export lantibiotic substrates from the membrane and/or cytoplasm of the cell. The Cpr system can confer resistance to multiple lantibiotics due to relaxed specificity for sensing substrates by the CprK histidine kinase, which results in activation of transcription from *Pcpr* as well as the ability of the ABC transporter components to recognize and transport multiple lantibiotic substrates.

anisms so that they can withstand antimicrobial peptides produced by the host. In the current work, we demonstrate that an intestinal pathogen has evolved a CAMP resistance mechanism that functions specifically as a means to avoid the toxic effects of bacterially produced antimicrobial peptides. *C. difficile* has apparently done so by coopting part of a lantibiotic regulatory and immunity system.

Many of the characterized lantibiotic synthesis and immunity systems are controlled by two-component positive regulatory systems (e.g., NisRK and SpaRK) that also regulate their own synthesis (34, 38). Like other lantibiotic two-component systems, the *cprR* response regulator (RR) appears to be activated by phosphorylation by the CprK sensor kinase (HK) (Table 2). But in lantibiotic systems, the RR and HK genes are genetically linked and cotranscribed, leading to increased production of both proteins upon induction (38). In contrast, the *cprR* and *cprK* transcripts are not linked and are not coordinately regulated. CprK and CprR are both expressed at low levels prior to lantibiotic exposure, but CprK expression is markedly induced upon activation while CprR expression is not (Table 2 and Fig. 3) (11). Thus, separation of these genes has allowed for differential expression of the two proteins. This differential expression results in greater induction of the histidine kinase, which may allow faster depletion of response regulator activity and, therefore, faster shutdown of *cprABC* transcription, when CAMPs are no longer present. In the subtilin system, immunity and toxin genes are autoinduced by mature subtilin and expression is also regulated by the stationary-phase regulators AbrB and SigH (39). We did not detect SigH binding sites for any of the *cpr* promoters, and the *cprABCK* and *cprR* transcripts are not differentially expressed in a *sigH* mutant (40). The genomic separation of *cprK* and *cprR* and the absence of regulation from stationary-phase controls may provide a means for uncoupling *cpr* ex-

pression from the conditions that stimulate lantibiotic producer systems. We are currently examining how CprK and CprR interact to regulate *cprABCK* expression *in vitro* to better understand how their genetic uncoupling affects CAMP resistance.

Aside from the uncoupling of CprR and CprK, the Cpr system both structurally and functionally mimics the subtilin immunity and regulation system, suggesting that the DNAs originated from a common ancestral source. The discovery that SpaK is able to effect a small negative change on CprR-mediated transcription when SpaK is activated by subtilin indicates that the sensor-response regulator interactions are mostly conserved as well. We analyzed the CprR and CprK of strain 630 for interaction/specificity-determining residues (as reported by Skerker et al. [41]) and compared these residues to the specificity regions of SpaR/SpaK (41). We found that CprK differed from SpaK at only 2/13 specificity-determining residues while CprR differed from SpaR at 3/10 specificity residues (data not shown). In contrast, when we compared the specificity regions of CprR/CprK to the two most-similar two-component systems of *C. difficile* (CD0481/CD0482 and CD0668/CD0669), we found between 5 and 10 deviations each. Thus, CprR and CprK interact in a manner more similar to that of SpaR and SpaK than to that of other *C. difficile* two-component systems.

The CprABC transporter provides resistance to multiple CAMPs, and *cprABC* expression is induced by a structurally diverse array of lantibiotic CAMPs produced by distantly related Gram-positive bacteria (outlined in Fig. 6). Using the information gained from the activation of *cprAp* by various lantibiotics, we performed a subtractive analysis to uncover which lantibiotic residues may be involved in activation of CprK. Of the type A lantibiotics that activate CprK, we identified a 5-residue motif that is

FIG 7 Subtractive analysis of CprK-activating lantibiotic residues. Structures of the lantibiotics that activate *cpr* transcription (34, 42–46, 49). Lantibiotic motifs and residues are coded as follows: red, meso-lanthione (Lan); blue, (2S,3S,6R)-3-methyl-lanthione (MeLan); purple, (2S,8S)-lysinoalanine; Dha, 2,3-didehydroalanine; Dhb, (Z)-2,3-didehydrobutyrine; Asp-OH, erythro-3-hydroxy-L-aspartic acid. Gray dotted lines designate the 5-residue motif that is common to all type A and type B lantibiotics that activate transcription from *cprAp*.

common to all of these structures (Fig. 7) (42–46). This motif spans from the meso-lanthione residue at position 7 through the methyl-lanthione bridge at residue 11 of the activating type A lantibiotics (i.e., ring A and ring B). This motif is common among type A lantibiotic structures (34). Ring A, in particular, has been shown to be important for autoinducing activity in the nisin system, suggesting that CprK and NisK recognize many of the same residues for activation (47). However, full activation of NisK requires the first three structural rings (A, B, and C) (48). The fact that CprK can be activated by lantibiotics with dissimilar residues outside the rings A and B while NisK cannot supports the evidence that lantibiotic specificity of producer sensors involves additional residues which are not required for activation of CprK. Because lantibiotic producers express toxin synthesis and immunity genes

concurrently upon sensor activation, it would be energetically costly to produce toxin in response to a non-self substrate. Thus, from an evolutionary perspective, narrow substrate recognition by lantibiotic producer strains allows for more-appropriate control of toxin production. Since *cpr* gene regulation is not tied to lantibiotic production, activation of this immunity mechanism by a wide range of substrates does not impose the cost of toxin production while still providing the benefits of immunity. Hence, *C. difficile* has reaped the benefits of multiple-lantibiotic immunity without the costs of lantibiotic production.

The ring A and ring B configuration of residues is not present in the type B lantibiotic cinnamycin, but based on the nuclear magnetic resonance (NMR) structural prediction of this compound (49), the stereo arrangement of the molecule aligns the meso-lanthione residue at position 4 and the methyl-lanthione bridge (residues 5 and 11) in combination with the glycine and proline residues (residues 8 and 9), similar to the configuration of the activating type A lantibiotics (Fig. 7, gray dotted lines). In contrast, the lantibiotics that did not activate CprK did not contain this arrangement of residues (35, 50). Accordingly, we propose that a combination of the lanthione group, methyl-lanthione bridge, and proline and glycine residues comprises the motif that interacts with CprK and leads to activation of the CprK-CprR two-component system.

The ability of the Cpr system to respond to multiple toxins produced by other bacteria may give *C. difficile* an important advantage when competing against bacteriocin-producing strains in the intestinal environment. Numerous bacteriocins are produced by the indigenous microbiota of the intestine (51–54). Though the production of bacteriocins can have significant effects on the ability of bacteria to compete within the host environment (55–57), very little is understood about the role of bacteriocin production in maintenance of gut homeostasis or colonization resistance to invading pathogens, such as *C. difficile*. Nonetheless, *C. difficile* infections are usually preceded by disruption of the intestinal microbiota, which results in drastic declines in bacterial diversity and complexity—including disruption of many lineages that can produce bacteriocins.

In addition to the *cprABC* CAMP resistance mechanism, the *C. difficile* 630 genome encodes at least ten other genetic loci with significant similarity to lantibiotic or bacteriocin immunity systems (CD0665-CD0669, CD0478-CD0482, CD0665-CD0669, CD0820-CD0824, CD0643-CD0646, CD1095-CD1099, CD0363-CD0368, CD0316-CD0320, CD1266-CD1270, and CD1752-CD1755). It is unclear what substrates activate these mechanisms, but the presence of multiple genetic mechanisms for bacteriocin immunity underscores their evolutionary importance for *C. difficile* in the host intestinal environment. An understanding of how the *cpr* and other bacteriocin resistance systems contribute to the fitness of *C. difficile* in the intestine may provide valuable insight for the prevention of colonization and disease. Additionally, because the Cpr system is capable of responding to many substrates, it may be useful for the testing and development of natural and modified bacteriocins as therapeutics to treat bacterial infections. In light of these findings, the future development of lantibiotic compounds as antimicrobials for the treatment of infections, especially those caused by *C. difficile*, should consider the development and spread of lantibiotic resistance traits an obvious risk.

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