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Identification of a Genetic Locus Responsible for Antimicrobial Peptide Resistance in *Clostridium difficile*▽

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Clostridium difficile **causes chronic intestinal disease, yet little is understood about how the bacterium interacts with and survives in the host. To colonize the intestine and cause persistent disease, the bacterium must circumvent killing by host innate immune factors, such as cationic antimicrobial peptides (CAMPs). In this study, we investigated the effect of model CAMPs on growth and found that *C. difficile* is not only sensitive to these compounds but also responds to low levels of CAMPs by expressing genes that lead to CAMP resistance. By plating the bacterium on medium containing the CAMP nisin, we isolated a mutant capable of growing in three times the inhibitory concentration of CAMPs. This mutant also showed increased resistance to the CAMPs gallidermin and polymyxin B, demonstrating tolerance to different types of antimicrobial peptides. We identified the mutated gene responsible for the resistance phenotype as CD1352. This gene encodes a putative orphan histidine kinase that lies adjacent to a predicted ABC transporter operon (CD1349 to CD1351). Transcriptional analysis of the ABC transporter genes revealed that this operon was upregulated in the presence of nisin in wild-type cells and was more highly expressed in the CD1352 mutant. The insertional disruption of the CD1349 gene resulted in significant decreases in resistance to the CAMPs nisin and gallidermin but not polymyxin B. Because of their role in cationic antimicrobial peptide resistance, we propose the designation *cprABC* for genes CD1349 to CD1351 and *cprK* for the CD1352 gene. These results provide the first evidence of a *C. difficile* gene associated with antimicrobial peptide resistance.**

Clostridium difficile is a major nosocomial pathogen that causes chronic intestinal disease that is both difficult and costly to treat (12, 53). A number of risk factors have been identified for infection with *C. difficile*, namely, antibiotic exposure, the disturbance of the normal colonic flora, advanced age, and hospitalization (11, 28, 38, 66). Though *C. difficile* is a strict anaerobe, it can survive outside the host intestinal environment as a dormant spore, which allows the bacterium to spread to other hosts via the fecal-oral route of infection (67). The progression of *C. difficile* disease is dependent on the ability of the bacteria to germinate from the spore form, multiply, reside, and produce toxins within the intestinal tract (37, 53, 69).

Aside from the principal toxins A and B, a few additional factors have been implicated in the colonization or virulence of *C. difficile.* These include the S-layer proteins (6), the Cwp84 protease (29), Cwp66 adhesin (68), the Fbp68 fibronectin-binding protein (21), CDT binary toxin (3, 57), and flagellar proteins (64). While these factors may play a role in the initial adherence and colonization of the bacteria, little is known about how *C. difficile* is able to survive the host immune response to cause persistent infections. There is evidence that host immune factors, such as alpha-defensins, can inhibit *C. difficile* toxin B activity, and they may play an important role in susceptibility to *C. difficile* disease (13). Diseases such as ulcerative colitis and Crohn's disease, which appear to be immune related, are linked to an increased risk of *C. difficile* infection, although the reason for this association is unknown (1, 25). Experiments in animal models of *C. difficile* disease have demonstrated that defects in the host innate immune response lead to greater colonization and disease progression (33). These studies underscore that the host immune response plays a key role in the clearance of *C. difficile* infection, but details of the host-pathogen interactions are not understood.

The intestinal tract is a dynamic organ that relies on many factors to prevent being overrun by pathogenic bacteria. The innate immune response in particular provides a constant and immediate line of host defenses that protect the intestines from invasion. The production of cationic antimicrobial peptides (CAMPs) represents a critical component of host defense that bacteria must overcome to cause disease (4, 16). CAMPs are small, positively charged peptides that are made by bacteria, fungi, plants, and animals, and they have microbicidal activities (15). Humans produce a variety of CAMPs, including defensins, cathelicidins, and thrombocidins, that accumulate in areas of the body that routinely encounter microorganisms, such as the intestines (42, 50). These compounds are able to kill bacteria directly, and many can stimulate the host to produce additional immune effectors that contribute to microbial death (17).

Resistance to antimicrobial peptides is essential for bacteria to establish persistent infections, and consequently many human bacterial pathogens are resistant to the killing effects of CAMPs (16, 43, 50). Because *C. difficile* causes chronic intestinal disease, we hypothesized that it has evolved mechanisms for defense against host antimicrobial peptides that allow it to colonize and persist in the intestinal environment. In the current work, we investigated the response of *C. difficile* to CAMPs *in vitro*. We found that the bacteria can adapt to the presence of these compounds and identified a broad-range

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TABLE 1. Bacterial strains and plasmids

Plasmid or strain	Relevant genotype or features	Source or reference
Strains		
E. coli		
HB101	F^- *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*	B. Dupuy
MC135	HB101 containing pRK24 and pMC123	This study
MC138	HB101 containing pRK24 and pMC125	This study
MC161	HB101 containing pRK24 and pMC147	This study
C. difficile		
630	Clinical isolate	71
JIR8094	Erm^s derivative of strain 630	44
CCUG37769	Serotype A4; $ToxA^+$, $ToxB^+$	Lars Burman
MC119	JIR8094 *cprK1*	This study
MC137	JIR8094 pMC123	This study
MC141	JIR8094 *cprA::ermB*	This study
MC146	MC141::Tn*916* (*cprABC*); Erm^r	This study
MC162	JIR8094 pMC147	This study
MC163	MC119 pMC147	This study
MC164	MC119 pMC123	This study
B. subtilis		
BS49	CU2189::Tn*916*	P. Mullany
MC144	BS49 Tn*916*::pMC130	This study
Plasmids		
pRK24	Tra^+, Mob^+; *bla*, *tet*	65
pCR2.1	*bla*, *kan*	Invitrogen
pUC19	Cloning vector; *bla*	72
pCE240	*C. difficile* TargeTron construct based on pJIR750ai (group II intron, *ermB*::RAM, *ltrA*); *catP*	C. Ellermeier
pSMB47	Tn*916* integrational vector; Cm^r, Erm^r	36
pJIR1456	*E. coli-C. perfringens* shuttle vector; *catP*	35
pMC122	pCE240 retargeted to *cprA*	This study
pMC123	*E. coli-C. difficile* shuttle vector; *bla*, *catP*	This study
pBL58	pMC123 with BsrGI and two HindIII sites removed	This study
pMC125	5.48 kb SfoI/SmaI fragment from pMC122 cloned in SmaI site of pMC123	This study
pMC130	2,945-bp DNA sequence corresponding to *cprABC* plus 500-bp upstream sequence cloned as BamHI/SphI fragment in pSMB47	This study
pMC147	pBL58 with 1,798 bp of CD1352 and upstream promoter region (P*cprK*)	This study

CAMP resistance mechanism and a component of its regulatory system. This is the first identified CAMP resistance mechanism in *C. difficile* and appears to be related to lantibiotic self-tolerance systems.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Clostridium difficile* strains were grown in BHIS medium (61) supplemented with 0.1% L-cysteine. Media for the growth of *C. difficile* were supplemented with 250 μg D-cycloserine ml^{-1}, 50 μg kanamycin ml^{-1}, 10 to 20 μg thiamphenicol ml^{-1}, 5 μg erythromycin ml^{-1}, or 5 μg erythromycin ml^{-1} and 50 μg kanamycin ml^{-1} as needed. *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. *Bacillus subtilis* strains were routinely grown at 37°C in L broth (34) or BHIS medium supplemented with 1 μg erythromycin ml^{-1} when needed. For the propagation of *B. subtilis* in the anaerobic chamber, the growth medium was supplemented with 5 mM KNO_3. *Escherichia coli* strains were grown at 37°C in L or BHIS medium supplemented with 20 μg chloramphenicol ml^{-1} or 100 μg ampicillin ml^{-1} as needed.

Bacterial strain and plasmid construction. Oligonucleotides used in this study are listed in Table 2. *C. difficile* strain 630 DNA, GenBank accession number AM180355 (58), was used as a template for PCR amplification unless otherwise noted. The sequencing of cloned DNA fragments was performed by the Tufts University Core Facility using an ABI 3130XL DNA sequencer. A null mutation in CD1349 (*cprA*) was created in several steps. First, plasmid pMC122 was created by the retargeting of the group II intron from pCE240 (kindly supplied by Craig Ellermeier, University of Iowa) using primers oMC123, oMC124, and oMC125 and the EBS Universal primer as outlined in the TargeTron users manual (Sigma-Aldrich), followed by the initial cloning of the retargeted fragment in pCE240 digested with BsrGI/HindIII. Plasmid pCE240 is a derivative of pJIR750ai (Sigma-Aldrich) that is similar to pMTL007 (19). pMC123 was constructed by the stepwise addition of three DNA fragments to pUC19. A 390-bp *oriT* fragment was amplified from pJIR1456 (35) using primers oMC15 and oMC16 and cloned as an EcoO1091/AatII fragment in pUC19. A 1,043-bp fragment containing *catP* from pJIR1456, amplified using primers oMC13 and oMC14, then was added as a PciI/SapI fragment. Lastly, a 3.1-kb plasmid origin of replication (*ori69*) amplified from *C. difficile* strain CCUG37769 using primers oMC122 and oMC123 was inserted at the SfoI site. The intron-containing part of pMC122 was excised as a 5.48-kb SfoI/SmaI fragment and cloned in the SmaI site of pMC123, creating pMC125, which was introduced by transformation into *E. coli* strain HB101(pRK24), resulting in strain MC138. pRK24 is a derivative of the broad-host-range plasmid RP4 that mobilizes IncP *oriT* plasmids. MC138 then was mated with *C. difficile* strain JIR8094, resulting in the transfer of pMC125 by conjugation as previously described (8), except that transconjugants were selected on BHIS plates supplemented with D-cycloserine, kanamycin, and thiamphenicol. The inactivation of CD1349 (*cprA*) was selected for by screening transconjugants for erythromycin resistance and thiamphenicol sensitivity, resulting in strain MC141. The insertional disruption of *cprA* was confirmed using primers oMC130 and oMC97, which are located outside the region of insertion in the *cpr* locus.

To complement the *cprA* disruption, a 2,945-bp fragment containing the *cprABC* operon and its upstream region was amplified using oMC146 and oMC147 as primers and was cloned between the BamHI and SphI sites of pSMB47, generating pMC130. MC144 was created by the integration of pMC130 into the chromosomal Tn*916* locus of *B. subtilis* strain BS49. MC146 is a

TABLE 2. Oligonucleotides

Primer	Sequence (5′→3′)	Use/location
oMC13	5′-GCACATGTCCTTGGTTGTGTTGCTTTTCG-3′	*catP* gene PCR
oMC14	5′-GCTCTTCTAGCGCCTACGGGGAATT-3′	*catP* gene PCR
oMC15	5′-GCAGGCCCTCGGATCTTTTCCGCTGCA–3′	*oriT* PCR
oMC16	5′-GCGACGTCCTTATCGGCCAGCCTCG-3′	*oriT* PCR
oMC44	5′-CTAGCTGCTCCTATGTCTCACATC-3′	*rpoC* qPCR
oMC45	5′-CCAGTCTCTCCTGGATCAACTA-3′	*rpoC* qPCR
oMC55	5′-GGTTTTCTAAATGGGAAGGTAAA–3′	*cprK* sequencing
oMC56	5′-CCAGATAAGTCATTAATTGCTGCG-3′	*cprK* sequencing
oMC57	5′-GCTCTGAATAACAGTTCTCTATCTA-3′	*cprK* sequencing
oMC96	5′-CGTTCAGGTCAATTCTCTCTAGGC-3′	*cprA* qPCR/sequencing
oMC97	5′-GGTCAAGACCATTTGTAGGCTC-3′	*cprA* qPCR/sequencing
oMC121	5′-CGTATTGGCGCCAACCAGGAATATAGTGTATGCA-3′	*ori69* PCR
oMC122	5′-CGTATTGGCGCCCTAGAGAACCAAACGACGG-3′	*ori69* PCR
oMC123[a]	5′-AAAAGCTTTTGCAACCCACGTCGATCGTGAAGAAAGGATTTATGTGCGC CCAGATAGGGTG-3′	*cprA* intron retargeting
oMC124[a]	5′-CAGATTGTACAAATGTGGTGATAACAGATAAGTCATTTATCATAACTTA CCTTTCTTTGT-3′	*cprA* intron retargeting
oMC125[a]	5′-CGCAAGTTTCTAATTTCGGTTCTTTCTCGATAGAGGAAAGTGTCT-3′	*cprA* intron retargeting
EBS universal	5′-CGAAATTAGAAACTTGCGTTCAGTAAAC-3′	Sigma-Aldrich
oMC126	5′-GCTACTACTTTATTGAGTACGGCA-3′	CD1348 qPCR
oMC127	5′-CTTCATCCTTCTTTACAACTGCTG-3′	CD1348 qPCR
oMC128	5′-GCGTATTACACAGGAGTTTGAACC-3′	*ori69* sequencing
oMC129	5′-GCATTGTATGTATCTTTTATTCCTAGC-3′	*ori69* sequencing
oMC130	5′-GGAGCAGATGGCAGTTGATAAC-3′	*cprA* PCR
oMC137	5′-GCTGCTTTCAACTGGTGGTA-3′	*cprB* qPCR
oMC138	5′-CCAGTCCAAACGTCTTTCATTTC-3′	*cprB* qPCR
oMC139	5′-GCACATTTGCATTGCTTTAATGGG-3′	*cprC* qPCR
oMC140	5′-CATTGAACACACAACACCTGAC-3′	*cprC* qPCR
oMC141	5′-GAAGAAAGGCATGCATATTCAGA-3′	*cprK* qPCR
oMC142	5′-TCATACCCAATGTCTCTGGT-3	*cprK* qPCR
oMC144	5′-GCGGATCCGGTTCGGAAAAGGAACATC-3	P*cprK* cloning
oMC145	5′- GCAAGCTTGTTACCATAGCACCACCAG-3′	P*cprK* cloning
oMC146	5′-GCGGATCCGCTACTACTTTATTGAGTACGG C-3′	P*cprABC* cloning
oMC147	5′-GCGCATGCCAAGCATTTGTACCCTGTCCTC-3′	P*cprABC* cloning
BC1a[b]	5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC TTCCGATCTAACCT-3′	DNA barcoding
BC1b[b,c]	5′-*GGTTAGATCGGAAGAGCGGTTCAGCAGGAATGC CGAGACCGATCTCGTATGCCGTCTTCTGCTTG-3′	DNA barcoding
Olj139	5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA-3′	DNA barcoding
Olj140	5′-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAAC-3′	DNA barcoding

[a] Underlined bases denote retargeted sequences.
[b] Underlined bases define the barcode.
[c] Asterisk denotes the position of 5′ phosphorylation.

transconjugant from the mating of *B. subtilis* strain MC144 and *C. difficile* strain MC141, resulting in the integration of the plasmid::Tn*916* fusions of the donor strains into the *C. difficile* chromosome, as previously described (18).

To investigate the impact of the overexpression of *cprK*, a plasmid-based copy of the *cprK* gene and its upstream region (P*cprK*) was introduced into strains of *C. difficile* as follows. A 1,798-bp fragment corresponding to *cprK* and the entire *cprC-cprK* intergenic region was amplified using primers oMC144 and oMC145 and cloned between the BamHI and HindIII sites of pBL58 to create pMC147. pBL58 is a derivative of plasmid pMC123 in which one HindIII site (outside the multiple cloning site [MCS]) and all BsrGI sites were removed (L. Bouillaut, personal communication). pMC147 then was introduced into *E. coli* strain HB101(pRK24) by transformation, resulting in strain MC161. MC161 then was mated with *C. difficile* strains JIR8094 and MC119 to transfer pMC147 by conjugation, resulting in strains MC162 and MC163, respectively. As a control, *E. coli* strain HB101(pRK24) was transformed with pMC123, yielding strain MC135. pMC123 then was transferred to *C. difficile* strains JIR8094 and MC119 by conjugation with MC135, creating strains MC137 and MC164, respectively.

MIC determination. Susceptibility tests were performed anaerobically in BHIS broth as follows. *C. difficile* strain JIR8094 and derivatives were grown to an optical density at 600 nm (OD_{600}) of 0.5 (exponential growth phase) and then diluted 1:100 into 1 ml of fresh medium to give an inoculum of approximately 5×10^5 CFU/ml. The medium was supplemented with a range of concentrations of nisin (MP Biomedicals), polymyxin B (≥6,000 USP U/mg; Sigma-Aldrich), gallidermin (Alexis Biochemicals), LL-37 (17-29)/FK-13 (cathelicidin) (Phoenix Pharmaceuticals), or Magainin II (Phoenix Pharmaceuticals), and cultures were incubated for 18 h at 37°C. The MIC was defined as the lowest concentration of antimicrobial peptide that prevented visible turbidity after 18 h.

qRT-PCR. Exponential-phase cultures of *C. difficile* grown in BHIS medium or BHIS supplemented with nisin, gallidermin, or polymyxin B were diluted 1:50 in BHIS or BHIS supplemented with the same antimicrobial peptides and incubated anaerobically at 37°C. Samples for RNA isolation were taken when cultures reached an OD_{600} of 0.4 (active growth), diluted with an equal volume of cold 1:1 ethanol-acetone, and stored at −80°C. RNA was extracted and treated to remove contaminating DNA as previously described (8). SuperScript II reverse transcriptase (Invitrogen) was used to generate randomly primed cDNA pools from 1 μg of RNA per sample, as instructed by the manufacturer. To control for chromosomal DNA contamination, mock cDNA synthesis reaction mixtures containing no reverse transcriptase were used as negative controls in subsequent amplifications. cDNA samples were diluted 4-fold and used as templates for quantitative, real-time PCR (qRT-PCR) of *rpoC* (primers oMC44/oMC45), *cprA* (oMC96/oMC97), *cprB* (oMC137/oMC138), *cprC* (oMC139/oMC140), *cprK* (oMC141/oMC142), and CD1348 (oMC126/oMC127), using Qiagen SYBR green PCR mix and an MXP3005 thermocycler (Stratagene/Agilent Technologies). Reactions were performed in a final volume of 25 μl using 4 μl of diluted cDNA and 1 μM each primer. Reactions were performed in triplicate using cDNA extracted from each of a minimum of two biological

TABLE 3. MICs

Drug	MIC (μg/ml) for strain:			
	630	MC119	MC141	MC146
Nisin	90	350	25	120
Polymyxin B	300	500	300	≥300
Gallidermin	0.5	≥1.3	≥0.3	0.6

replicates, and results are presented as the means and standard deviations of these experiments. Amplification included 40 cycles of the following steps: 30 s at 95°C, 60 s at 50°C, and 30 s at 72°C. Results were calculated using the comparative cycle threshold method (56), in which the amount of target mRNA is normalized relative to an internal control transcript (*rpoC*). The two-tailed Student's *t* test was used to analyze the data.

Identification of a chromosomal mutation through SNP analysis. Purified genomic DNA of strain MC119 was sheared, barcoded, and amplified for high-throughput DNA sequencing as previously described (7a). Deep sequencing was performed using an Illumina Genome Analyzer II by the Tufts University Core Facility. Sequenced genomic DNA reads were aligned to the *C. difficile* 630 genome (NCBI accession number NC_009089) using MAQ 0.6.6 (http://maq.sourceforge.net). Reads with more than two mismatches from their aligned positions were removed from the assembly. A consensus sequence was called from the assembled reads, and single-nucleotide polymorphisms (SNPs) and short insertions and deletions were identified. The list was filtered using MAQ with the following parameters: a minimum read depth of 3, a minimum consensus quality of 20, and a minimum adjacent consensus quality of 20.

RESULTS

Adaptation of *C. difficile* to nisin. To understand the effects of CAMPs on the colonization and virulence of *C. difficile*, we first determined the sensitivity of *C. difficile* to CAMPs and its ability to adapt to the presence of these molecules. As a model, we first tested the CAMP nisin, a commercially available CAMP produced by *Lactococcus lactis* that is routinely used to study the effects of CAMPs on bacteria (5, 7, 10, 31). The MIC of nisin for strain JIR8094 was approximately 90 μg/ml (Table 3). Based on the MIC, we then assessed the growth effects of a range of sublethal nisin concentrations. As shown in Fig. 1A, increasing the concentration of nisin in the medium resulted in an increasing delay in logarithmic growth.

Many bacterial species have been shown to adapt to low concentrations of CAMPs by inducing the expression of CAMP resistance mechanisms. The induction of these resistance mechanisms allows the bacteria to grow in previously inhibitory concentrations (14, 47, 54). We found that preconditioning *C. difficile* to inhibitory, but not lethal, levels of nisin (30 μg/ml) enabled the bacteria to grow in otherwise-inhibitory concentrations (Fig. 1B). Cells that were preconditioned in nisin at 30 μg/ml and then diluted into fresh medium containing nisin at the same concentration were able to grow without a lag and at the same rate as wild-type cells grown without nisin. When preconditioned cells were subcultured without nisin and then reexposed to nisin, however, growth again was inhibited (Fig. 1C). These results demonstrate that preconditioning allows cells to grow in nisin due to transient adaptation and not to mutation.

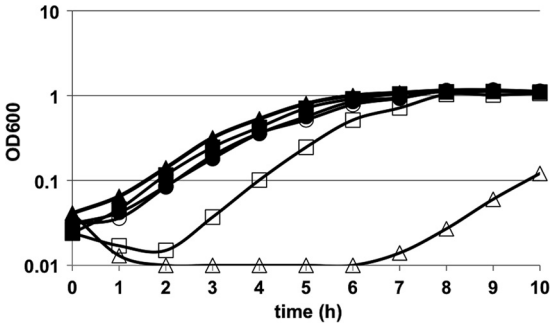

FIG. 2. Growth of *cpr* mutants in nisin. *C. difficile* wild-type (JIR8094, squares), MC119 (*cprK* W235C, circles), and MC141 (*cprA::*intron::ermB, triangles) cells grown in BHIS medium (filled shapes) or BHIS medium supplemented with 10 μg/ml nisin (open shapes).

Isolation and identification of a nisin-resistant mutant. The ability of *C. difficile* to adapt to CAMPs most likely is accomplished by the induction of genes encoding resistance mechanisms. To identify such genes, we isolated spontaneous CAMP-resistant *C. difficile* mutants by plating wild-type cells on BHIS agar supplemented with nisin. Nisin-resistant colonies that appeared on plates containing 225 μg nisin per ml were tested for their ability to grow in BHIS broth with increasing concentrations of nisin. One such mutant, MC119, was able to grow in approximately four times the nisin MIC for the wild-type without preconditioning (Table 3). In addition, this mutant grew at wild-type rates in sublethal concentrations, a condition that delays the growth of wild-type bacteria (Fig. 2). MC119 also

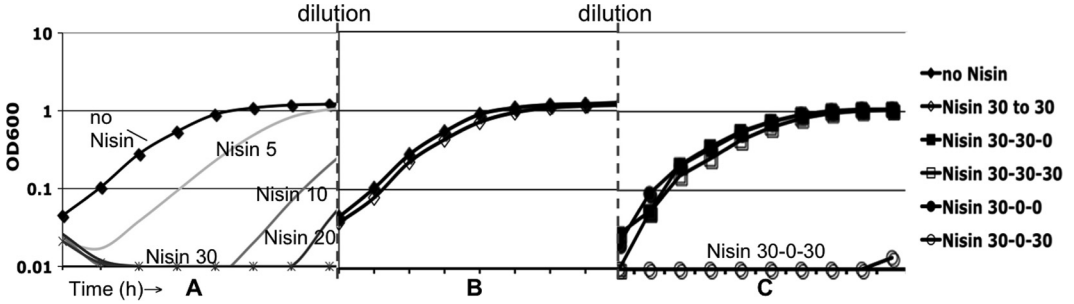

FIG. 1. Growth and adaptation of *C. difficile* to nisin. Cells were grown in BHIS medium with or without nisin as indicated (μg/ml). (A) *C. difficile* growth is inhibited by increasing concentrations of nisin. (B) *C. difficile* cells preconditioned in nisin at 30 μg/ml are able to grow at normal rates when resuspended in medium without nisin (30-0) or with nisin at 30 μg/ml (30-30). (C) Cells preconditioned in nisin maintain the ability to grow in nisin at the same concentration (30-30-30), but cells preconditioned in nisin (30) and then grown without nisin (30-0) lose the ability to grow in nisin without significant lag (30-0-30).

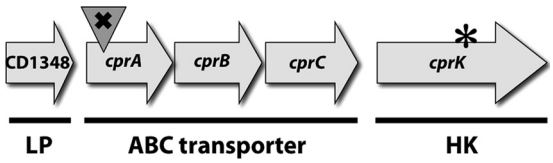

FIG. 3. Putative ABC transporter system and surrounding genes (CD1348 to CD1352) in JIR8094. CD1348 lipoprotein (LP), the CD1349 to CD1351 ABC transporter ABC-binding cassettes and permeases (*cprABC*), and orphan histidine kinase (HK) and CD1352 (*cprK*) are shown. × indicates the insertional disruption of *cprA*; * denotes a W235C spontaneous mutation in *cprK*.

showed increased resistance to the CAMPs gallidermin and polymyxin B, demonstrating tolerance to a variety of antimicrobial peptides (Table 3).

To map the CAMP resistance mutation in MC119, we resequenced the entire genome (41), the only method currently available to map spontaneous mutations in *C. difficile*. Using this approach, we identified four single-nucleotide polymorphisms that were represented at greater-than 85% frequency in the MC119 genome compared to that of the parent strain, JIR8094. These mutations were located in the coding sequences of genes CD1352 (100%), CD2125 (100%), CD2667 (85%), and CD3089 (95%). Five additional independently isolated mutants were obtained that also were resistant to high levels of nisin. Because the phenotypes of these mutants were identical to that of MC119, we sequenced the CD1352 gene of each and found that all had a mutation within CD1352. Because there was a 100% correlation between the high-nisin-resistance phenotype and mutations in the CD1352 gene, we further investigated this correlation. The mutation in MC119 lies within gene CD1352 and resulted in a tryptophan-to-cysteine alteration at amino acid position 235 (W235C), whereas the other five mutants had serine-to-tyrosine substitutions at amino acid 230 (S230Y). Based on the genome annotation (58), CD1352 is predicted to encode a lantibiotic sensor histidine kinase. Concordantly, by protein prediction modeling (SOSUI) (23, 39, 40), the CD1352 protein product appears to have all of the characteristics of a sensor histidine kinase (HK) member of a two-component regulatory system (TCS). The mutated amino acids at positions 230 and 235 are predicted to be located on the cytoplasmic side of the molecule, which includes the kinase-phosphatase and protein interaction regions, near but not within the catalytic active site (24). TCSs typically contain a sensor histidine kinase that receives an extracellular stimulus and transmits a signal to a partner protein, the response regulator (RR). The activated RR acts by binding DNA to regulate gene transcription (24). In most cases, the cognate HK and RR genes are located adjacently to each other on the chromosome, but there is no RR encoded near CD1352. If CD1352 is in fact an HK involved in regulating the transcription of CAMP resistance genes through a cognate RR, then this RR must be encoded elsewhere on the chromosome.

Effects of the CD1352 mutation on transcription of CD1348 to CD1352. As CD1352 appears to be a regulatory gene, we next sought to identify the potential target(s) of its regulation. CD1352 lies immediately downstream of a gene cluster that putatively encodes an ABC transport system (Fig. 3). This putative transporter and the CD1352 histidine kinase both share homology to lantibiotic immunity mechanisms, which are found in bacteria that produce such antimicrobial peptides (e.g., nisin) and are highly specific for individual peptides (55, 70). To test whether the CD1348-CD1351 cluster is regulated by the CD1352 gene product, we performed real-time quantitative PCR analysis of the CD1349 to CD1351 transcripts in the wild-type and MC119 mutant strains grown in the presence or absence of CAMPs. As shown in Fig. 4, levels of CD1349, CD1350, and CD1351 mRNA increased when wild-type cells were grown in the presence of nisin or gallidermin at sublethal concentrations. Moreover, the level of expression of these genes was higher in cells grown with nisin at 20 μg/ml than in cells grown at 10 μg/ml. Increased expression also was seen when cells were grown in sublethal concentrations of gallidermin (Fig. 5D to F). As predicted, the ABC transporter genes were very highly expressed in the MC119 mutant, even in the absence of CAMPs (Fig. 4). The expression of the CD1352 histidine kinase also increased in the wild-type strain during growth in nisin (Fig. 4D) or gallidermin (Fig. 5F) and was highly expressed in the presence or absence of CAMPs in the MC119 mutant, indicating that CD1352 also is regulated in response to CAMPs. These data demonstrated that the CD1349-CD1352 gene cluster is induced in response to CAMPs in wild-type *C. difficile* and raised the possibility that the overexpression of these genes contributes to the high CAMP resistance of the MC119 mutant. We therefore propose the designation *cprABC* for genes CD1349 to CD1351 and *cprK* for the CD1352 gene (*cpr* is a mnemonic for cationic peptide resistance).

ABC transporter systems often work in association with a lipoprotein carrier; auspiciously, a predicted lipoprotein is encoded by the upstream gene, CD1348 (Fig. 3). We tested the level of the transcription of CD1348 in the presence and absence of nisin, gallidermin, and polymyxin B, and we found no change in gene expression in the wild-type or MC119 (Fig. 4 and 5), indicating that this gene is not induced in response to these CAMPs and is not regulated by the *cprK* histidine kinase.

Effects of disruption of the *cprABC* operon on CAMP resistance. To test whether the *cprABC* genes play a role in CAMP resistance, we introduced an insertion mutation in the first gene of the operon, *cprA*, thereby creating a polar disruption of the *cprABC* operon. To do so, we used a TargeTron-based group II intron (27), which we retargeted for integration into *cprA* (see Materials and Methods). The resulting strain, MC141, then was tested for its ability to grow in the presence of nisin, polymyxin B, or gallidermin. The MICs of nisin and gallidermin, but not for polymyxin B, for MC141 exhibited significant decreases (Table 3). We also tested the resistance of the wild-type, MC119, and MC141 strains against two CAMPs of animal origin, LL-37 and Magainin II, and we found that all of the strains tested were resistant to the highest concentrations of these compounds tested (greater than 100 μM and 100 μg/ml, respectively); as a result, the animal CAMPs were not used in further analyses.

Figure 2 illustrates the considerable growth delay seen for MC141 exposed to nisin (10 μg/ml) compared to the growth of the wild type and the MC119 mutant. A similar delay in growth was observed when MC141 was grown in gallidermin (data not shown). Although MC141 had a pronounced lag when grown

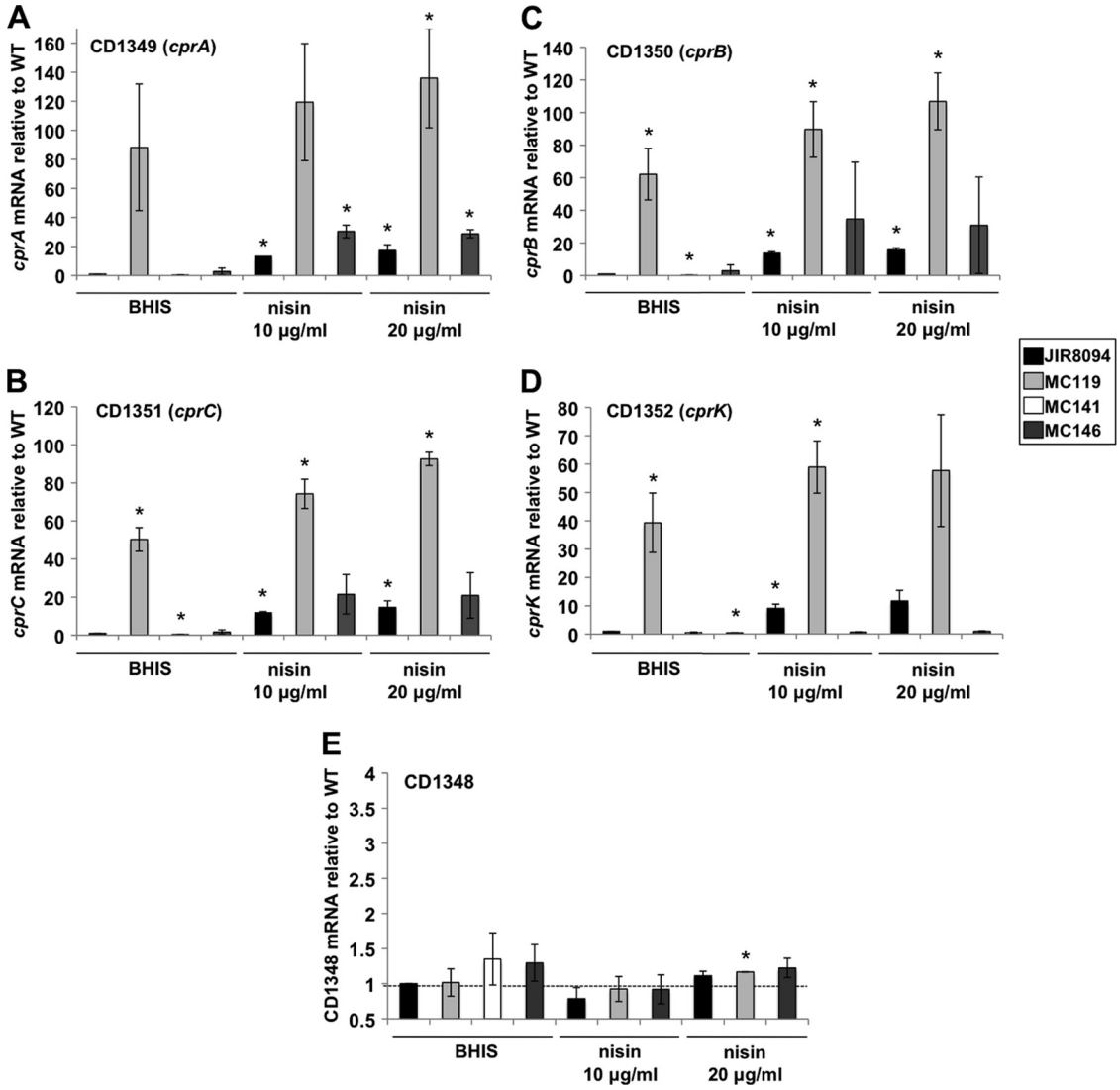

FIG. 4. qRT-PCR analysis of *cprA*, *cprB*, *cprC*, *cprK*, and CD1348 expression during growth in nisin. *C. difficile* wild-type (JIR8094), MC119 (*cprK* W235C), MC141 (*cprA*::intron::ermB), and MC146 (*cprA*::intron::*ermB*, P*cprABC*) strains were grown in BHIS supplemented with 0, 10, or 20 μg/ml nisin to an OD_{600} of 0.4 as indicated. RNA was harvested, cDNA synthesized, and qPCR performed using gene specific primers for *cprA* (A), *cprB* (B), *cprC* (C), *cprK* (D), and CD1348 (E). Results were normalized to an internal control gene (*rpoC*) and are presented as the ratio of each transcript level relative to wild-type and no-nisin controls. The means and standard deviations of biological replicates are shown (*, $P \leq 0.05$ by Student's *t* test).

in CAMPs, it did eventually grow at wild-type rates at sublethal concentrations of nisin and gallidermin (less than 20 and 0.1 μg/ml, respectively). This result implies that other CAMP resistance mechanisms can be induced and provide some compensation for the absence of *cpr* expression.

As expected, the transcription of the *cprA*, *cprB*, and *cprC* genes was markedly decreased in the MC141 mutant compared to levels for the wild type (Fig. 4), suggesting that these genes are cotranscribed as part of an operon. The expression of *cprK* in MC141, however, was at wild-type levels in the absence of CAMPs, indicating that *cprK* expression is not disrupted by the polar effects of the insertion in the *cprA* gene.

To confirm that the CAMP resistance defect of MC141 was due to the disruption of the putative ABC transporter system, we complemented MC141 with the native version of the CD1349-CD1351 region, including a 500-bp upstream region predicted to contain the promoter for these genes. The *cprABC* genes and the upstream region were integrated into the Tn*916* locus of *B. subtilis* strain BS49 (MC144) and transferred via conjugation to *C. difficile* strain MC141, creating a *cprABC*-complemented strain, MC146 (see Materials and Methods). The MICs for MC146 and growth in CAMPs were similar to those of the wild type (Table 3). In the wild type and in MC146, the expression of *cprABC* was induced during growth in nisin and gallidermin, but *cprK* transcription did not increase for MC146 (Fig. 4D and 5F). The disruption of the *cprABC* genes does not disrupt *cprK* expression, although both the *cprABC* mutant and *cprABC-trans*-complemented strains no longer induce the expression of *cprK* in CAMPs, suggesting that *cprK* is transcribed from its own promoter as well as from an upstream

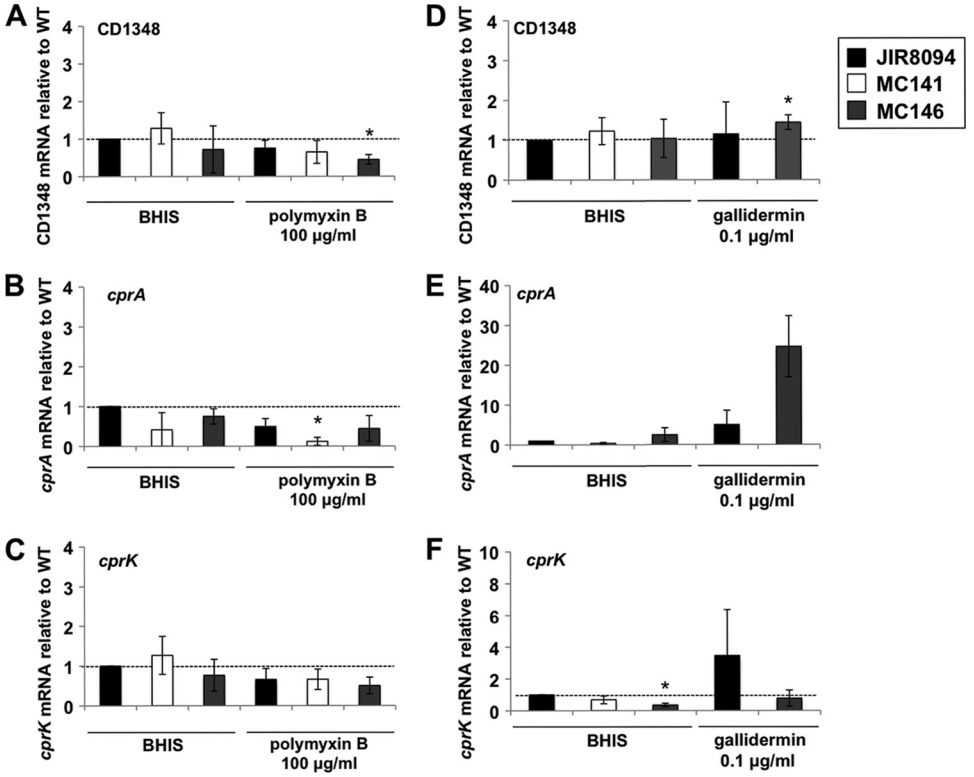

FIG. 5. qRT-PCR analysis of CD1348, *cprA*, and *cprK* expression during growth in polymyxin B or gallidermin. *C. difficile* wild-type (JIR8094), MC141 (*cprA*::intron::*ermB*), and MC146 (*cprA*::intron::*ermB*, P*cprABC*) strains were grown in BHIS alone or BHIS supplemented with 100 μg/ml polymyxin B or 0.1 μg/ml gallidermin to an OD_{600} of 0.4 as indicated. RNA was harvested, cDNA synthesized, and qPCR performed using gene-specific primers for CD1348 (A and D), *cprA* (B and E), or *cprK* (C and F). Results were normalized to an internal control gene (*rpoC*) and graphed as the ratio of each transcript level relative to wild-type and no-nisin controls. The means and standard deviations of biological replicates are shown (*, $P \leq 0.05$ by Student's *t* test).

promoter for *cprABC*, although this has not been verified. In addition, the *B. subtilis* strain MC144, which contains the *cprABC* locus within Tn*916*, also had increased resistance to nisin (data not shown). These data imply that the *cpr* locus is directly involved in resistance to multiple CAMPs.

Unlike the case for nisin and gallidermin, the growth of the *cprABC* mutant, MC141, in polymyxin B was similar to that of the wild type (Table 3). In contrast, the polymyxin B MIC for the *cprK* mutant, MC119, was higher than that for the wild-type parent (500 versus 300 μg/ml). The expression analysis of the *cprABC* and *cprK* genes during growth in a sublethal concentration of polymyxin B (100 μg/ml) demonstrated that neither the *cprABC* nor *cprK* gene are induced under these conditions (Fig. 5A to C). In fact, all of the strains, including MC119 (data not shown), appeared to have the decreased expression of *cprABC* in the presence of polymyxin B. Thus, the *cprABC*-encoded resistance mechanism does not contribute to resistance to polymyxin B, but *cprK* may regulate the transcription of an additional, as-yet unknown CAMP resistance mechanism.

Impact of wild-type *cprK* overexpression on transcription of *cpr* genes. To further investigate the role of *cprK* in the regulation of the *cpr* genes, an extrachromosally replicating, plasmid-based copy of the *cprK* gene and its upstream region (pMC147) was introduced into the wild-type and MC119 strains of *C. difficile*. The parent vector, pMC123, also was introduced into the wild-type and MC119 strains to control for variability due to plasmid replication and growth in the presence of the selective antibiotic thiamphenicol. Wild-type and MC119 strains carrying pMC123 (MC137 and MC164) or pMC147 (MC162 and MC163) then were grown in BHIS medium supplemented with 10 μg/ml thiamphenicol, with or without the addition of 10 μg/ml nisin. All of these strains grew at the same rate in the absence of nisin, and their growth in nisin paralleled the growth observed for the parent strains, with the exception of MC162 (JIR8094 pMC147), which had a more-than 2-h delay compared to the rate of the vector control strain (MC137) (data not shown). As anticipated, the introduction of a plasmid-borne copy of *cprK* resulted in modestly higher expression of *cprK* in MC162 than in MC137 in the absence of nisin (Fig. 6A). The expression of *cprA* was reduced in the MC162 strain in the absence of nisin, suggesting that an excess in *cprK* in a wild-type background results in the more tightly controlled transcription of the *cpr* genes, although the statistical significance of this result is uncertain (Fig. 6B). As suggested by the growth observed in nisin, the expression of the *cprA* transcript was unchanged in the MC119 mutant containing the plasmid copy of *PcprK* (MC163) compared to that of MC119 with vector alone (MC164). These data indicate that the wild-type *cprK* allele, even in multicopy, is recessive to the mutant version of *cprK* in MC119, leading us to conclude that the *cprK* mutation results in a gain-of-function defect. Because

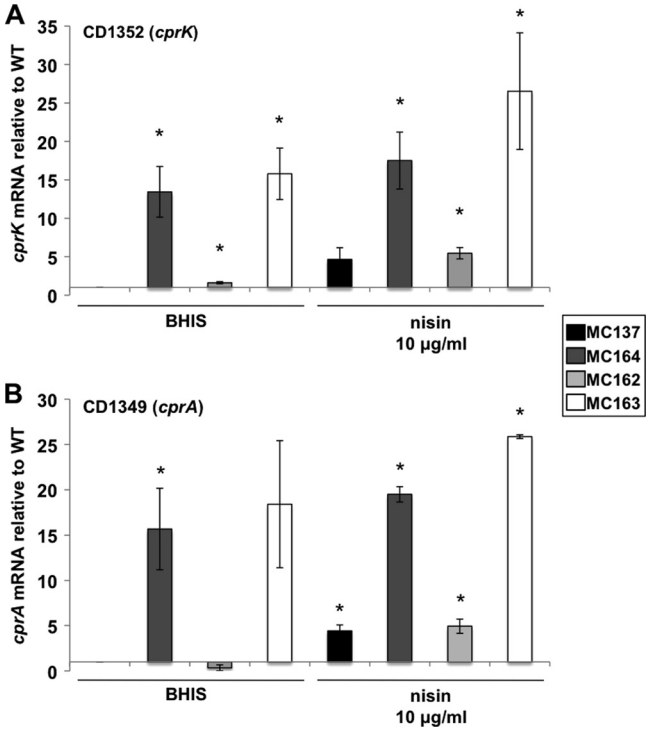

FIG. 6. qRT-PCR analysis of *cprA* and *cprK* expression with the addition of plasmid-encoded *cprK*. *C. difficile* wild-type and MC119 strains carrying control vector pMC123 (MC137 and MC164, respectively) or pMC147 (MC162 and MC163) were grown in BHIS medium supplemented with 10 μg/ml thiamphenicol with or without the addition of 10 μg/ml nisin to an OD_{600} of 0.4. RNA was harvested, cDNA synthesized, and qPCR performed using gene-specific primers for *cprK* (A) or *cprA* (B). Results were normalized to an internal control gene (*rpoC*) and graphed as the ratio of each transcript level to that of *rpoC* and then normalized to the wild type grown without nisin. The means and standard deviations of biological replicates are shown (*, $P \leq 0.05$ by Student's *t* test).

the phenotype of the MC119 gain-of-function mutation leads to the greater expression of the *cpr* operon, we can conclude that CprK functions as a positive regulator of *cprABC* expression in the presence of nisin.

DISCUSSION

CAMPs are critical components of the innate immune defense against bacterial pathogens. Accordingly, resistance to these peptides is a demonstrated virulence factor for many bacterial pathogens (16, 43, 48, 50). In this work, we show that *C. difficile*, like some other bacterial pathogens (14, 47, 54), is able to adapt to antimicrobial peptides by increasing the expression of CAMP resistance genes.

The principles of CAMP resistance are similar among different bacterial species, but the mechanisms for achieving resistance vary considerably. Mechanisms of CAMP resistance identified in bacteria include increasing the net positive charge of the cell wall or membrane (repulsion) (10, 49), the proteolytic cleavage of CAMPs (degradation) (60), sequestration by proteins that bind CAMPs (trapping) (26, 32), and efflux pumping (export) (59). By selecting a mutant resistant to nisin, we isolated a strain that can grow in the presence of elevated concentrations of multiple CAMPs (nisin, gallidermin, and polymyxin B) due to a mutation in a predicted histidine kinase (*cprK*). We also identified a nearby putative ABC transporter operon, designated *cprABC*, that is directly involved in resistance to nisin and gallidermin (but not polymyxin B) and is regulated by CprK. The *cprABC* transporter operon and the putative histidine kinase encoded by *cprK* have sequence similarity to lantibiotic immunity systems found in bacteria that make lantibiotics (e.g., nisin), suggesting that resistance to CAMPs is accomplished through the export of the peptides by the ABC transporter. The fact that the *cprABC* mechanism can provide resistance to both nisin and gallidermin and, to a lesser extent, polymyxin B, is highly unusual, as lantibiotic immunity mechanisms are highly specific for individual peptides (55, 70) and cross-immunity is very rare and of limited range (2, 9, 20). Limited cross-immunity has been demonstrated for the immunity proteins of some bacteria that produce structurally similar lantibiotics (2, 20, 22). Low-level immunity to lacticin 3147 can be conferred by overexpressing transporter proteins from *Bacillus licheniformis* and *Enterococcus faecium* in an *L. lactis* background, although the resistance effects of these genes in their natural hosts are not clear (9). The native immunity mechanisms for the CAMPs tested (nisin, gallidermin, and polymyxin B) do not exhibit cross-immunity (22, 46). In contrast, the *C. difficile cprABC* and *cprK* CAMP resistance genes do not appear to be associated with a lantibiotic synthesis gene cluster, can protect against multiple CAMPs, and provide significant resistance to these compounds.

The immunity genes for lantibiotics and other bacteriocins are diverse but often are composed of an ABC transporter system with or without an additional distinct immunity gene (55). The immunity gene product may provide protection independently of the ABC transporter or function synergistically with the transporter to generate resistance (22, 45, 51). For example, immunity to the lantibiotic subtilin is conferred by an ABC transporter system (SpaFEG) in conjunction with the lipoprotein immunity factor SpaI, encoded in the gene order *spaIFEG* on the *B. subtilis* chromosome (30, 63). Separately, SpaI and SpaFEG confer partial immunity; both are required for full immunity to subtilin. A predicted lipoprotein, CD1348, also is encoded upstream of the *cprABC* genes. Unlike the case for *spaI*-like genes that typically are increased during lantibiotic production (52, 55, 62), no change in CD1348 expression was detected in the wild type or MC119, indicating that this gene is not induced in response to these CAMPs. Our results, however, do not rule out the possibility that CD1348 provides or is part of an independent, constitutively expressed mechanism of resistance to CAMPs.

The most likely scenario is that the extracellular domain of CprK senses CAMPs, and as a result either the kinase or phosphatase activity of the cytoplasmic domain becomes activated. A response regulator that controls the *cprABC* operon and other genes whose identities are not known then becomes altered in activity, resulting in the induction of *cprABC* and other CAMP resistance genes. It is evident that *cprK* is a positive regulator of *cprABC* and expression in response to CAMPs and also is autoregulated. We have attempted to create an insertional disruption in *cprK* to further define its role as a positive or negative regulator of the *cpr* genes, but these attempts thus far have been unsuccessful. As there are no

apparent RR genes near *cprK* on the chromosome, the presumed RR is encoded at another location. The *C. difficile* genome encodes 51 putative response regulators, most of which are uncharacterized (58).

A BLAST homology search for the *cprABC* operon revealed homologs of these genes in all of the other *C. difficile* isolates sequenced to date, suggesting that this operon encodes a universal mechanism of CAMP resistance for the species. The fact that a *cprABC*-disrupted mutant (MC141) can eventually adapt, without additional mutations, to sub-MIC levels of nisin indicates that other, unidentified CAMP resistance mechanisms also are present in *C. difficile.* The implications of CAMP resistance in *C. difficile* may include resistance to CAMPs produced by the host innate immune system, resistance to bacteriocins and lantibiotics produced by indigenous bacteria, and resistance to food preservation processes involving bacteriocins. Considering the importance of CAMPs in defending against infection and the public health threat posed by *C. difficile*, the resistance mechanisms of these bacteria may present a target for the design of anti-infective therapeutics.

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