Published in final edited form as:

Anaerobe. 2015 April ; 32: 98–104. doi:10.1016/j.anaerobe.2015.01.002.

An Alkaline Phosphatase Reporter for use in *Clostridium difficile*

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Abstract

Clostridium difficile is an anaerobic, Gram-positive pathogen that causes severe gastrointestinal disease in humans and other mammals. *C. difficile* is notoriously difficult to work with and, until recently, few tools were available for genetic manipulation and molecular analyses. Despite the recent advances in the field, there is no simple or cost-effective technique for measuring gene transcription in *C. difficile* other than direct transcriptional analyses (e.g., quantitative real-time PCR and RNA-seq), which are time-consuming, expensive and difficult to scale-up. We describe the development of an *in vivo* reporter assay that can provide qualitative and quantitative measurements of *C. difficile* gene expression. Using the *Enterococcus faecalis* alkaline phosphatase gene, *phoZ*, we measured expression of *C. difficile* genes using a colorimetric alkaline phosphatase assay. We show that inducible alkaline phosphatase activity correlates directly with native gene expression. The ability to analyze gene expression using a standard reporter is an important and critically needed tool to study gene regulation and design genetic screens for *C. difficile* and other anaerobic clostridia.

Keywords

Clostridium difficile; alkaline phosphatase; reporter; *phoZ*; XP; pNP; AP; BCIP

1. INTRODUCTION

Although *Clostridium difficile* is a major nosocomial pathogen that causes severe gastrointestinal disease, the molecular mechanisms that control pathogenesis in this bacterium are poorly understood. The primary reasons that *C. difficile* remains an understudied pathogen are that the bacterium has long been difficult to cultivate and genetically manipulate. First, *C. difficile* is a fastidious organism and a strict anaerobe. Thus, for optimal and consistent growth, *C. difficile* is typically cultivated in rich growth medium

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within a strict anaerobic environment. Culture conditions notwithstanding, one of the greatest impediments has been the shortage of genetic tools for use in *C. difficile*.

Many of the molecular biology tools and techniques that are used to genetically manipulate other species have been difficult to adapt for *C. difficile*. However, techniques for studying *C. difficile* molecular biology have been developed in the past decade, including methods for gene disruption and plasmid transfer, random mutagenesis strategies and the application of next-generation sequencing technologies (1–7). Despite the advancements in molecular technologies over the past decade, genetic analysis of *C. difficile* remains a challenge.

The constraints of an anaerobic growth environment have been particularly problematic for the adaption of gene reporter technologies used in other bacteria. Reporter gene fusions are incredibly useful tools in the study of gene regulation and signal transduction, defining regulatory elements and promoters, and for the development of genetic screens (8). The most commonly used reporter gene fusions include colorimetric reporters, such as β -galactosidase (*lacZ*) and β -glucuronidase (*gusA*), fluorescent proteins (*e.g.*, *gfp*, *dsRed*, *mCherry*), and chemiluminescent reporters (*i.e.*, luciferase; *luxAB*). Unfortunately, these reporters require oxygen for proper reporter protein folding or full enzymatic activity (9–12) (data not shown), and consequently have little utility in anaerobic bacteria. Antibiotic-based reporters, such as Chloramphenicol Acetyl Transferase (*cat*) and neomycin (*neo*), are excluded because *cat* is one of the few selectable markers available and because *C. difficile* has high intrinsic resistance to aminoglycosides. A cyan fluorescent protein (CFP) reporter (13), a mCherry reporter (14), and anaerobically-functioning fluorescent reporters have been described, including SNAP (15) and LOV-based fusions (16, 17). While these fluorescent reporters are useful, visualization of activity can be difficult due to photo-bleaching and to high variability of fluorescence within a population of cells (13, 15–17). β -glucuronidase (*gusA*) reporters have been used in the heterologous host, *Clostridium perfringens*, an anaerobe that is somewhat aerotolerant, to measure *C. difficile*-specific gene expression (18). In addition, *gusA* reporters have been used in *C. difficile* (19); however, these assays exhibit low sensitivity and are cost-prohibitive.

While other methods are available for quantifying *C. difficile* gene transcription, including qRT-PCR, microarray and RNA-seq analyses, these techniques are expensive and require an investment in specialized equipment and enzymes. Since these methods are expensive and time-consuming, it is not practical to employ these techniques in large-scale genetic screens. In contrast, colorimetric reporter assays are relatively inexpensive and require a low investment in materials and reagents. In this study, we evaluate the alkaline phosphatase gene, *phoZ*, from *Enterococcus faecalis* as a transcriptional reporter for use in *C. difficile*. We report that *C. difficile* can express an active *phoZ* transcriptional fusion, which functions as a sensitive and quantifiable reporter of gene expression. This alkaline phosphatase reporter provides *C. difficile* researchers with a versatile new tool for assessing gene expression.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured at 37°C in LB (20) or BHIS medium (21) aerobically or anaerobically, and supplemented with 20 µg chloramphenicol ml⁻¹ or 100 µg ampicillin ml⁻¹, as needed. *Enterococcus faecalis* was cultured on BHI or BHIS medium as previously described (22). *Clostridium difficile* strains were cultured at 37°C in an anaerobic chamber as previously detailed (23, 24). *C. difficile* strains were grown in BHIS medium supplemented with 2–10 µg thiamphenicol ml⁻¹, 5 µg erythromycin ml⁻¹, 0.5–5 µg nisin ml⁻¹ or 100–400 µg XP (BCIP, 5-Bromo-4-chloro-3-indolyl phosphate; Sigma-Aldrich) ml⁻¹ as indicated.

2.2. Strain and plasmid construction

E. faecalis strain V583 (GenBank accession AE016830) was used as a template for *phoZ* amplification. Oligonucleotides used for PCR and qRT-PCR are listed in Table 2. Isolation of plasmid DNA, PCR and cloning were performed using standard protocols. The 1477 bp *phoZ* coding sequence and upstream ribosomal binding site were amplified using primers oMC829/oMC830. This fragment was cloned into the *Bam*HI and *Sph*I sites of pMC123 (4) to create pMC358 (Figure 1A). pMC368 was created by cloning the *PcprA* fragment from pMC211 (25) as *Eco*RI/*Bam*HI into pMC358. pMC375 was created by amplifying a 600 bp fragment upstream of *dltD* with oMC850/oMC823 and cloning this fragment as *Eco*RI/*Bam*HI into pMC358. pMC370 was created by cloning the *phoZ* fragment from pMC358 as *Bam*HI/*Sph*I into pSMB47 (Figure 1B). To construct pMC371, the *PcprA::phoZ* insert was amplified from pMC368 using primers oMC909/oMC830, followed by digestion and cloning as a *Sal*I/*Sph*I fragment into pSMB47. Note that the *cat* cassette of pSMB47 is only functional as a selectable marker in *E. coli*. Sequencing of cloned DNA fragments was performed by Eurofins MWG Operon. Plasmid maps were created using SnapGene Viewer software.

pMC358, pMC368 and pMC375 were introduced into *E. coli* strain MC101 by transformation, resulting in strains MC445, MC461 and MC468, respectively. MC445, MC461 and MC468 were conjugated independently to *C. difficile* strain 630 *erm* as previously described (26, 27) using 10 µg thiamphenicol ml⁻¹ to select for the plasmids in *C. difficile* and 50 µg kanamycin ml⁻¹ to counterselect against *E. coli* after conjugation.

pMC370 and pMC371 were integrated into the *Tn916* region within the chromosome of *B. subtilis* strain BS49 and selected for on BHIS plates containing 5 µg erythromycin ml⁻¹ as previously described (28, 29), except that 50 µg kanamycin ml⁻¹ was added to counterselect against *B. subtilis* after conjugation.

2.3 Alkaline Phosphatase Assays

Assays of alkaline phosphatase (AP) activity in *C. difficile* were adapted from previously described assays with some modifications (30, 31). To begin, overnight *C. difficile* cultures were back-diluted to an OD₆₀₀ of approximately 0.05 in BHIS medium (21) with or without addition of nisin, as indicated. Duplicate (2 ml) samples of each culture were taken when the

cells reached an OD₆₀₀ of approximately 0.5. Samples were pelleted and the supernatant discarded, then stored at -20°C. To prepare samples for assay, cell pellets were first washed with 0.5 ml of cold Wash buffer (10 mM Tris-HCl, pH 8.0, 10 mM MgSO₄), pelleted and resuspended in 1 ml Assay buffer (1 M Tris-HCl, pH 8.0, 0.1 mM ZnCl₂). 500 µl of cell suspension was transferred to a separate tube for alkaline phosphatase tests. 300 µl of additional Assay buffer, 50 µl of 0.1% SDS and 50 µl of chloroform were added to the suspension, which was then vortexed for 1 min and incubated at 37°C for 5 min. Sample tubes were then placed on ice for 5 min to cool. To begin the assay, samples were pre-warmed to 37°C, then 100 µl of 0.4% pNP (*p*-nitrophenyl phosphate in 1M Tris-HCl, pH 8.0; Sigma-Aldrich) was added to each sample at 10 second intervals. A blank without cells was prepared as a negative control in all experiments. Samples were inverted to mix and placed in a 37°C water bath. Upon development of a light yellow color, 100 µl of stop solution (1 M KH₂PO₄) was added and tubes placed in an ice bath to stop the phosphatase reaction. Time elapsed (min) for the assay was recorded. Assay samples were then centrifuged at max speed for 5 minutes. Absorbance for each sample was read at OD₄₂₀ and OD₅₅₀. Units of activity were calculated and normalized to cell volume as follows:

$$\frac{[\text{OD}_{420} - (1.75 \times \text{OD}_{550})]1000}{t(\text{min}) \times \text{OD}_{600} \times \text{vol. cells}(\text{ml})}$$

Technical duplicates were performed and averaged for each AP assay set. A minimum of three biological replicates were performed for each experiment. The results are presented as the means and standard error of the experimental replicates. The two-tailed Student's *t* test was used to compare the results of the variable and control sets as indicated.

2.4 Quantitative reverse transcription PCR analysis (qRT-PCR)

Cultures for qRT-PCR analysis were harvested in parallel with the alkaline phosphatase assay samples described above. Cultures were harvested into cold 1:1 ethanol:acetone prior to RNA purification as previously described (4, 32). Total RNA was DNase I treated and cDNA synthesized as previously described (33). qRT-PCR primers were designed using the IDT PrimerQuest tool and primer efficiencies were determined for each primer set (<http://www.idtdna.com/Scitools/Applications/Primerquest>). Technical triplicates were performed for each qPCR reaction set for a minimum of three biological replicates. Relative quantification of transcription was performed using the internal control transcript, *rpoC*, for normalization. The transcriptional ratios of variable and control sets are presented as the means and standard error of the means for the data obtained. The two-tailed Student's *t* test was used to compare the ratios of the variable and control sets as indicated.

3. RESULTS AND DISCUSSION

3.1. Evaluation of alkaline phosphatase activity and construction of vectors for alkaline phosphatase reporter fusions

Before evaluating the use of *phoZ* as a reporter, we first assessed the potential for inherent alkaline phosphatase (AP) activity in *C. difficile*. A protein BLAST search revealed no proteins with significant homology to *phoZ* within the genome of any *C. difficile* strain. For good measure, *C. difficile* strains 630 *erm* and R20291 were grown overnight on BHIS agar medium with and without the chromogenic substrate XP as described in the Materials and

Methods. No apparent blue color development of the colonies was detected following overnight anaerobic growth or aerobic incubation of the plates, indicating that *C. difficile* does not produce significant levels of alkaline phosphatase under the tested conditions (data not shown).

In order for *phoZ* to be useful as an anaerobic reporter, the PhoZ produced under anaerobic conditions needs to be enzymatically active. To determine whether the *E. faecalis phoZ* gene product was functional when produced under anaerobic conditions, *E. faecalis* was grown on BHIS agar with and without XP, anaerobically and aerobically. *E. faecalis* grown aerobically exhibited the blue colony phenotype indicative of AP activity, while the bacteria that were incubated anaerobically appeared white until the plates were allowed to develop in the presence of oxygen (data not shown). However, when cells expressing *phoZ* were assayed anaerobically using the AP substrate *pNP*, significant color change was observed, indicating that PhoZ is enzymatically active anaerobically (data not shown). Thus, the AP protein folds properly anaerobically, but oxidation is required for the XP cleavage product to produce the color-forming precipitate as previously described (34). Accordingly, AP reactions using the *pNP* substrate can be performed under anaerobic conditions, but reactions using the XP substrate will not produce a colored precipitate without further oxidation.

To investigate *phoZ* as a transcriptional reporter in *C. difficile*, two independent reporter constructs were created. The first was an extrachromosomally-replicating plasmid containing the *phoZ* reporter gene. The *phoZ* gene was amplified from *E. faecalis* and cloned into the *C. difficile-E.coli* shuttle vector pMC123, creating pMC358 (Figure 1A). This vector does not have a promoter to drive the expression of *phoZ*, thus no alkaline phosphatase activity should be produced in strains harboring this plasmid. To enable expression of *phoZ* in *C. difficile* the strong promoter, *PdltD*, or the nisin-inducible promoter, *Pcpr*, was cloned into pMC358 upstream of *phoZ* to give pMC375 and pMC368, respectively (4, 35, 36). All three plasmids were conjugated into *C. difficile* strain 630 *erm* as described in the Materials and Methods.

A second *phoZ* vector was created to permit single-copy chromosomal-based reporter fusions. To this end, *phoZ* and *Pcpr::phoZ* were amplified and cloned independently into the plasmid pSMB47 to create pMC370 (Figure 1B) and pMC371, respectively. pSMB47 can replicate in *E. coli*, but integrates into the chromosome of *B. subtilis* strain BS49 at the *Tn916* locus and can be conjugated via *Tn916* onto the chromosome of *C. difficile* (37–39).

3.2. Alkaline phosphatase is expressed and functional in *C. difficile*

To investigate the activity of alkaline phosphatase expressed in *C. difficile*, strains carrying the plasmid-based *Pdlt::phoZ* (MC499), the promoterless *phoZ* control (MC448) or the empty vector (MC324) were grown on BHIS plates containing XP and thiamphenicol. After overnight anaerobic growth, plates were removed from the anaerobic chamber and alkaline phosphatase activity was allowed to develop aerobically. As shown in Figure 2, *C. difficile* containing the *Pdlt::phoZ* fusion turn blue on XP plates. The development of color in colonies was visible within 20 minutes of removing the plate from the anaerobic chamber. Cells carrying the empty vector or the promoterless *phoZ* vector remained white on XP

plates. These results demonstrate that the *E. faecalis* PhoZ is active when produced by *C. difficile*.

3.3. Alkaline phosphatase transcriptional reporter activity correlates directly with gene-specific transcription

To determine the amount of alkaline phosphatase activity in *C. difficile* cells expressing *phoZ*, quantitative AP assays were performed. This AP assay was based on previously published assays with minor modifications (30, 31) (see Materials and Methods). *C. difficile* strains expressing the promoterless *phoZ* (MC448) and the *Pdlt::phoZ* fusion (MC499) from a plasmid were used to assess the expression and activity of alkaline phosphatase. Each strain was grown in BHIS broth, and culture samples were taken during mid-logarithmic growth ($OD_{600} = \sim 0.5$) for AP assays. The strain expressing the *Pdlt::phoZ* fusion exhibited a 144-fold increase in AP activity compared to the promoterless *phoZ* construct, indicating that a *C. difficile*-specific promoter can robustly drive measurable *phoZ* expression (Figure 3A).

To further determine the sensitivity and linear range of the *phoZ* promoter, we used the inducible *cpr* promoter (4). *C. difficile* strains expressing either the promoterless *phoZ* integrated on the chromosome (MC488) or from a plasmid (MC448), or the *Pcpr::phoZ* fusion on the chromosome (MC489) or from a plasmid (MC486) were grown in BHIS broth alone or with the addition of 0.5 μg or 5 μg ml^{-1} nisin. Culture samples were later taken during mid-logarithmic growth ($OD_{600} = \sim 0.5$) for AP assays and RNA preparation. Shown in Figure 3B, strains expressing the promoterless *phoZ* from the chromosome or from a plasmid exhibit very low alkaline phosphatase activity. As expected, strains expressing either *Pcpr::phoZ* fusion had higher AP activity when grown in nisin (~ 10 – 20 -fold in the presence of 0.5 μg ml^{-1} nisin and ~ 30 – 50 -fold in the presence of 5 μg ml^{-1} nisin), demonstrating that the *cprA* promoter is induced in a dose-dependent manner in the presence of nisin, as observed previously (4, 35). In addition, AP activity is significantly lower from the *PcprA::phoZ* fusion (MC486) in BHIS medium (1.3 AP units) compared to the constitutive, stronger *Pdlt::phoZ* fusion (MC499; 26 AP units; compare Figure 3B to 3A). Altogether, these results indicate that the *phoZ* reporter can be used to accurately quantify gene expression in a wide dynamic range with little basal expression.

To determine if AP activity from the *PcprA::phoZ* constructs correlates with native gene expression, qRT-PCR analysis was performed to measure chromosomal *cprA* gene expression. Expression of native *cprA* was increased ~ 100 -fold in the presence of 0.5 μg ml^{-1} nisin and ~ 170 – 200 -fold in the presence of 5 μg ml^{-1} nisin (Figure 4). To compare chromosomal *cprA* expression to the AP activity measured from the *PcprA::phoZ* fusion in the absence and presence of nisin, we determined the fold change in AP activity and transcript levels in various conditions (Table 3). The fold change in *PcprA::phoZ* fusion AP activity and in native *cprA* transcription is similar between the chromosomal and plasmid reporter constructs, although the plasmid reporter constructs are more sensitive (Table 3; **bolded numbers**). In addition, this data demonstrates that the presence of an additional *cprA* promoter in the chromosome or on a plasmid does not affect native gene expression or its

regulation as *cprA* transcription is equal between all strains in the absence or presence of nisin (qRT-PCR data in Table 3; Figure 4).

Our data demonstrate that *phoZ* can function as an alkaline phosphatase reporter to provide qualitative or quantitative analysis of gene expression in *C. difficile*. This *phoZ* reporter will be amenable to multiple uses, including the analysis of promoter function, identifying transcriptional regulatory effects, and genetic screens. For genetic screens performed using XP substrate, it is critical to replica plate the bacteria anaerobically prior to the removal of cells from the chamber, as brief exposure to oxygen can kill vegetative *C. difficile*. In addition measuring *phoZ* reporter activity in sporulating cells may require additional extraction techniques, such as the use of French pressure cell press, for complete cell lysis and accurate measurement of alkaline phosphatase activity. Notably, the use of the reporter on a multi-copy plasmid will facilitate increased expression for analyzing low levels of gene expression or screening for a range of regulatory effects in a variety of conditions. As with any construct expressed on a plasmid, additional considerations must be acknowledged when interpreting the results (e.g. titration of regulatory factors and plasmid burden).

It is important to note that we normalized alkaline phosphatase activity to the optical density of cultures at the time cells were harvested. Because of cell lysis, the optical density of *C. difficile* cells after resuspension in assay buffer was inconsistent and unreliable. However, some strains or mutants could exhibit aberrant cellular morphology that would result in an inaccurate correlation between optical density readings and the number of cells present. In this case, alkaline phosphatase activity can be normalized to total cellular protein (mg ml⁻¹), which can be determined by precipitating cells with trichloroacetic acid, to remove cross-reacting or interfering substances, and performing a standard protein quantification assay (e.g. bicinchoninic acid assay).

CONCLUSIONS

The use of a standardized reporter for analysis of gene expression and regulatory effects as well as the development of genetic screens is an effective molecular biology tool for studying prokaryotes. Thus, the development of a colorimetric and quantifiable reporter system that functions well in an anaerobe will be useful for the study of *C. difficile* molecular genetics. The *phoZ* reporter is a simple, critically-needed system that will allow cost-effective and time-efficient genetic analysis in *C. difficile* and other anaerobic organisms.

ACKNOWLEDGEMENTS

We give special thanks to Charles Moran for helpful critiques of the manuscript and to Jeremy Boss for use of the BioRad CFX96 Real Time PCR Detection System. This research was supported by the U.S. National Institutes of Health through research grants DK087763, DK101870 and AI109526 to S.M.M.; KLN and RAP were supported through training grants AI106699 and GM099644, respectively. The content of this manuscript is solely the responsibility of the authors and does not necessarily reflect the official views of the National Institutes of Health.

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HIGHLIGHTS

- We describe an effective transcriptional reporter assay for use in *C. difficile*.
- Promoter::*phoZ* fusion activity correlates with native gene expression *in vivo*.
- The *phoZ* reporter is a simple method for quantifying gene expression.

Figure 1. Overview of alkaline phosphatase reporter constructs
Plasmid maps of pMC358 (for construction of plasmid *phoZ* reporters) and pMC370 (for construction of chromosomal *phoZ* reporters).

Figure 2. Visualization of alkaline phosphatase activity from *C. difficile* grown on agar plates *C. difficile* strains A) MC324 (empty vector), B) MC448 (promoterless *phoZ*) and C) MC499 (*Pdlt::phoZ*) were grown anaerobically overnight on BHIS agar plates containing 400 μg XP substrate ml^{-1} and 2 μg thiamphenicol ml^{-1} . Plates were removed from the anaerobic chamber and development of color was monitored over 24 hours ("H" indicates the number of hours after removal from the anaerobic chamber). Visible color development was observed within 20 minutes of aerobic atmospheric exposure.

Figure 3. Alkaline phosphatase activity from the *cprA::phoZ* constructs is increased in the presence of nisin

(A) *C. difficile* strains MC448 (promoterless *phoZ* on a plasmid) and MC499 (*PdlA::phoZ* on a plasmid) were grown to an $\text{OD}_{600} = \sim 0.5$ in BHIS medium. (B) *C. difficile* strains MC488 (promoterless *phoZ* on the chromosome), MC489 (*PcprA::phoZ* on the chromosome), MC448 (promoterless *phoZ* on a plasmid) and MC486 (*PcprA::phoZ* on a plasmid) were grown to an $\text{OD}_{600} = \sim 0.5$ in BHIS medium in the presence or absence of nisin. Alkaline phosphatase activity was determined as described in the Materials and Methods. The means and standard errors of the means of three biological replicates are shown.

Figure 4. Native *cprA* transcription correlates with *PcprA::phoZ* alkaline phosphatase activity
qRT-PCR analysis of *cprA* expression in *C. difficile* strains MC488 (promoterless *phoZ* on the chromosome), MC489 (*PcprA::phoZ* on the chromosome), MC448 (promoterless *phoZ* on a plasmid) and MC486 (*PcprA::phoZ* on a plasmid) grown to an OD₆₀₀ = ~0.5 in BHIS medium in the presence or absence of nisin. The means and standard errors of the means of three biological replicates are shown.

Table 1**Bacterial Strains and plasmids**

Plasmid or Strain	Relevant genotype or features		Source, construction or reference
Strains			
<i>E. faecalis</i>			
V583	clinical isolate		(40, 41)
<i>E. coli</i>			
HB101	F- <i>mcrB mrr hsdS20</i> (τ_B γ μ_B) <i>recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20</i>		B. Dupuy
MC101	HB101 pRK24		B. Dupuy
MC445	HB101 containing pRK24 and pMC358		This study
MC461	HB101 containing pRK24 and pMC368		This study
MC468	HB101 containing pRK24 and pMC375		This study
<i>B. subtilis</i>			
BS49	CU2189:: <i>Tn916</i>		P. Mullany
MC472	BS49 <i>Tn916</i> ::pMC370		This study
MC473	BS49 <i>Tn916</i> ::pMC371		This study
<i>C. difficile</i>			
R20291	Clinical isolate		
630	Clinical isolate		(42)
630 <i>erm</i>	Erm ^S derivative of strain 630		N. Minton (43)
MC324	630 <i>erm</i> pMC123		(25)
MC448	630 <i>erm</i> pMC358		This study
MC486	630 <i>erm</i> pMC368		This study
MC488	630 <i>erm</i> <i>Tn916</i> :: <i>phoZ</i>		This study
MC489	630 <i>erm</i> <i>Tn916</i> :: <i>PcprA</i> :: <i>phoZ</i>		This study
MC499	630 <i>erm</i> pMC375		This study
Plasmids			
pRK24	Tra+, Mob+; <i>bla</i> , <i>tet</i>		
pUC19	Cloning vector; <i>bla</i>		(44)
pSMB47	<i>Tn916</i> integrational vector; CmR, ErmR		(45) (GenBank U69267)
pMC123	<i>E. coli</i> - <i>C. difficile</i> shuttle vector; <i>bla</i> , <i>catP</i>		(37)
pMC211	pMC123 <i>PcprA</i>		(25)
pMC358	pMC123 <i>phoZ</i>		This study
pMC368	pMC123 <i>PcprA</i> :: <i>phoZ</i>		This study
pMC370	pSMB47 <i>phoZ</i>		This study
pMC371	pSMB47 <i>PcprA</i> :: <i>phoZ</i>		This study
pMC375	pMC123 <i>Pdlt</i> :: <i>phoZ</i>		This study

Table 2

Oligonucleotides

Primer	Sequence (5' → 3')	Use/location
oMC44	5'-CTAGCTGCTCCTATGTCTCACATC-3'	<i>rpoC</i> qPCR (CD0067) (4)
oMC45	5'-CCAGTCTCTCCTGGATCAACTA-3'	<i>rpoC</i> qPCR (CD0067) (4)
oMC96	5'-CGTTCAGGTCAATTCTCTTAGGC-3'	<i>cprA</i> qPCR (CD1349) (4)
oMC97	5'-GGTCAAGACCATTGTAGGCTC-3'	<i>cprA</i> qPCR (CD1349) (4)
oMC823	5'-gccggatccATTTTCTCTCCTCTAAAAATATTCAA -3'	<i>PdltD</i> cloning (CD2854)
oMC829	5'-gccggatccGTCAATGTATGGGTAGATATGAAGG -3'	<i>phoZ</i> cloning (EF2973)
oMC830	5'-gcgcatgcCGTTCTGCTTTTCTTCATTTTG -3'	<i>phoZ</i> cloning (EF2973)
oMC850	5'-gcggaattcTTCTTATATACCATCTGAAATACAGG -3'	<i>PdltD</i> cloning (CD2854)
oMC901	5'-CTGAAGCGAAGGCAACTGAA-3'	<i>phoZ</i> qPCR (EF2973)
oMC902	5'-GCTTGCTGTCCGACCAAATA-3'	<i>phoZ</i> qPCR (EF2973)
oMC909	5'-GCCgtcgacGACATGGAAGTAGAAGTTAAGG-3'	<i>PcprA</i> cloning

Table 3

Fold change of *cprA* gene expression analyzed by alkaline phosphatase activity and qRT-PCR in *C. difficile* grown with and without nisin.

Strain	Relevant Genotype	Nisin 0.5 $\mu\text{g ml}^{-1}$ BHIS ^{a,d}		Nisin 5 $\mu\text{g ml}^{-1}$ BHIS ^{b,d}		Nisin 5 $\mu\text{g ml}^{-1}$ AP		Nisin 0.5 $\mu\text{g ml}^{-1}$ AP	
		AP	qRT-PCR	AP	qRT-PCR	AP	qRT-PCR	AP	qRT-PCR
MC488	Tn916: <i>phoZ</i>	1.1 $\pm$ 0.1	89.3 $\pm$ 10.5	1.0 $\pm$ 0.1	162.7 $\pm$ 11.7	1.0 $\pm$ 0.0	1.8 $\pm$ 0.1	1.0 $\pm$ 0.0	1.8 $\pm$ 0.1
MC489	Tn916::P <i>cprA</i> :: <i>phoZ</i>	17.3 $\pm$ 1.1	84.7 $\pm$ 2.6	32.5 $\pm$ 7.9	193.0 $\pm$ 27.1	2.7 $\pm$ 0.3	2.3 $\pm$ 0.4	2.7 $\pm$ 0.3	2.3 $\pm$ 0.4
MC448	<i>pphoZ</i>	1.2 $\pm$ 0.1	110.0 $\pm$ 8.3	1.5 $\pm$ 0.2	201.3 $\pm$ 18.0	1.2 $\pm$ 0.1	1.8 $\pm$ 0.1	1.2 $\pm$ 0.1	1.8 $\pm$ 0.1
MC486	pP <i>cprA</i> :: <i>phoZ</i>	23.3 $\pm$ 2.9	78.5 $\pm$ 5.4	48.3 $\pm$ 1.4	152.0 $\pm$ 19.0	2.2 $\pm$ 0.3	1.9 $\pm$ 0.2	2.2 $\pm$ 0.3	1.9 $\pm$ 0.2

^a Fold change of alkaline phosphatase (AP) activity or transcript levels (qRT-PCR) in cells grown in 0.5 $\mu\text{g ml}^{-1}$ nisin to BHIS medium with no supplement.

^b Fold change of alkaline phosphatase (AP) activity or transcript levels (qRT-PCR) in cells grown in 5 $\mu\text{g ml}^{-1}$ nisin to BHIS medium with no supplement.

^c Fold change of alkaline phosphatase (AP) activity or transcript levels (qRT-PCR) in cells grown in 5 $\mu\text{g ml}^{-1}$ nisin to 0.5 $\mu\text{g ml}^{-1}$ nisin.

^d The means and standard errors of the means of three biological replicates are shown.