

# Construction and Characterization of a Lactose-Inducible Promoter System for Controlled Gene Expression in *Clostridium perfringens*<sup>∇</sup>

Andrea H. Hartman, Hualan Liu, and Stephen B. Melville\*

Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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*Clostridium perfringens* is a Gram-positive anaerobic pathogen which causes many diseases in humans and animals. While some genetic tools exist for working with *C. perfringens*, a tightly regulated, inducible promoter system is currently lacking. Therefore, we constructed a plasmid-based promoter system that provided regulated expression when lactose was added. This plasmid (pKRAH1) is an *Escherichia coli*-*C. perfringens* shuttle vector containing the gene encoding a transcriptional regulator, BgaR, and a divergent promoter upstream of gene *bgaL* (*bgaR*-P<sub>*bgaL*</sub>). To measure transcription at the *bgaL* promoter in pKRAH1, the *E. coli* reporter gene *gusA*, encoding β-glucuronidase, was placed downstream of the P<sub>*bgaL*</sub> promoter to make plasmid pAH2. When transformed into three strains of *C. perfringens*, pAH2 exhibited lactose-inducible expression. *C. perfringens* strain 13, a commonly studied strain, has endogenous β-glucuronidase activity. We mutated gene *bglR*, encoding a putative β-glucuronidase, and observed an 89% decrease in endogenous activity with no lactose. This combination of a system for regulated gene expression and a mutant of strain 13 with low β-glucuronidase activity are useful tools for studying gene regulation and protein expression in an important pathogenic bacterium. We used this system to express the *yfp-pilB* gene, comprised of a yellow fluorescent protein (YFP)-encoding gene fused to an assembly ATPase gene involved in type IV pilus-dependent gliding motility in *C. perfringens*. Expression in the wild-type strain showed that YFP-PilB localized mostly to the poles of cells, but in a *pilC* mutant it localized throughout the cell, demonstrating that the membrane protein PilC is required for polar localization of PilB.

*Clostridium perfringens* is an important bacterial pathogen, causing gas gangrene, food poisoning, necrotic enteritis, and other diseases in humans and many others in agriculturally important livestock (3, 11, 21, 22). Strains that are transformable by electroporation have been isolated and their genomes sequenced (18, 22, 25, 32), and many groups have developed effective methods to create site-directed and random mutants (7, 10, 14, 31). However, other genetic tools available for studying genes are limited or absent, including an inducible promoter system, although they have been developed for use in *Clostridium acetobutylicum* (6, 28). A well-regulated promoter system would be a valuable tool in *C. perfringens* for complementing mutants, controlling the expression of fusion-tagged proteins, and modulating the intracellular levels of proteins to near physiological conditions.

There are several characteristics which are desirable in an inducible promoter system. Ideally, a promoter system would work in a variety of *C. perfringens* strains, since different diseases are caused by strains that are genetically highly diverse (18). It would be easy to induce, not requiring a temperature shift, expensive chemicals, or specific media. It would have a large range of activity based on the concentration of inducer added and low activity in the absence of inducer. These characteristics would allow a protein to be highly expressed for potential purification or analysis of the effects of overexpression on the phenotype of the strain. Tight regulation allows the introduction of potentially lethal genes as well as observation

of phenotypic changes that occur in the absence of expression of a gene that has been inactivated by mutation. Finally, a rapid response to the presence of the inducer would be desirable in studying short-term effects of the presence of the gene product on the cell. An example of a plasmid-based system that fulfills all of these criteria is the pBAD family of plasmids developed by Guzman and coworkers for use in *E. coli* (8). The plasmids employ the AraC protein to provide arabinose-inducible expression from the divergently transcribed promoter for the *araBAD* operon (8).

We used the genome sequence of *C. perfringens* strain 13 to identify regulators and promoters that, like the pBAD plasmids, would likely fulfill the desired requirements. We identified a lactose-inducible regulatory system, consisting of a gene encoding a transcriptional regulator, *bgaR*, and the promoter of a divergently transcribed regulated gene, *bgaL*, and constructed a plasmid-based system for controlled gene expression that functions in three different pathogenic strains of *C. perfringens*. We used the system in one of these strains to express a yellow fluorescent protein (YFP)-tagged type IV pilus-associated ATPase (PilB) and showed that cellular localization of this protein was dependent on a membrane-bound partner protein, PilC.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains, plasmids, and primers used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium supplemented with antibiotics as needed (400 μg/ml erythromycin, 100 μg/ml ampicillin, or 20 μg/ml chloramphenicol) and agar for plates. *C. perfringens* was grown in a Coy anaerobic chamber in brain heart infusion (BHI) medium (Difco) or on BHI plates with the appropriate antibiotics as indicated (30 μg/ml erythromycin or 20 μg/ml chloramphenicol). PY medium (30 g/liter

\* Corresponding author. Mailing address: 218 Life Science I, Washington Street, Virginia Tech, Blacksburg, VA 24061. Phone: (540) 231-1441. Fax: (540) 231-4043. E-mail: melville@vt.edu.

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description, relevant phenotype or genotype, or sequence (5' to 3')	Source or reference
<b>Strains</b>		
<i>E. coli</i> DH10B	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS mcrBC</i> ) φ80 <i>dlacZ</i> ΔM15 <i>lacX74 deoR recA1 araD139</i> Δ( <i>ara, leu</i> )7697 <i>galU galK</i>	Gibco/BRL
<i>C. perfringens</i>		
13	Gangrene strain	C. Duncan
SM101	Acute food poisoning strain	32
JGS4143	Chicken necrotic enteritis strain	J. G. Songer
AH1	<i>bglR</i> ::pAH4, from strain 13	This study
AH2	<i>bgaR</i> ::pAH6, from strain 13	This study
SM126	<i>pilC</i> mutant derived from strain 13	30
<b>Plasmids</b>		
pGEM-T Easy	Ampicillin resistance	Promega
pSM240	Contains <i>cpe-gusA</i> ; chloramphenicol resistance	9
pAH1	Mutated <i>bgaR</i> with P <sub>bgaL</sub> in pSM240	This study
pAH2	pSM240 with <i>bgaR</i> and P <sub>bgaL</sub> upstream of <i>cpe-gusA</i>	This study
pKRAH1	Contains <i>bgaR</i> -P <sub>bgaL</sub> and polylinker; chloramphenicol resistance	This study
pSM300	Suicide plasmid in <i>C. perfringens</i> ; erythromycin resistance	29
pAH3	Contains a fragment of strain 13 <i>bglR</i> in pGEM-T Easy	This study
pAH4	Contains a fragment of strain 13 <i>bglR</i> in pSM300	This study
pAH5	Contains a fragment of strain 13 <i>bgaR</i> in pGEM-T Easy	This study
pAH6	Contains a fragment of strain 13 <i>bgaR</i> in pSM300	This study
pAH7	Contains strain 13 <i>bgaR</i> and P <sub>bgaL</sub> in pGEM-T Easy	This study
pSW4-YFP	Vector containing a low GC codon-optimized <i>yfp</i> gene	23
pAH11	Contains <i>yfp-pilB</i> in pGEM-T Easy	This study
pKRAH-Y	pKRAH1 with the <i>yfp</i> gene	
pKRAH-YB	pKRAH1 with the <i>yfp-pilB</i> fusion gene	
<b>Primers</b>		
OAH1	GTTAACCTCGAGATGAAAAGTATTAGGGC	
OAH2	GCAGTTCTGCAGTATCTTCATGGTATTC	
OAH5	GGAGAGGTACCAGTTGAACATAAGGGAGG	
OAH6	CATGGAGCTCTCTTATCTCTAGAACTAATTC	
OAH13	CCGAGCTCTTACCCCGGGATC	
OAH14	CCGCGGTAATAACAAAAAGGAGAACGCATAATGTCAAAAAGGAG	
OAH36	CACACGGAATGGATGAATTATATAAGATGAAATATACCATTAAAGAT ATAGATATGAAGC	
OAH37	TTTCATATCTATCTTTAATGGTATATTTTCATCTTATATAATTCATCCATTCCGTGTGTAATCC	
OAH38	GAAGCGAGCTCTTTAAACCCAAACATCTAAC	
OKR055	GAATCTAGAATCCGCGGTAGTCGACATCCATGGTTGAGCTCATGGATCCTAA	
OKR056	AGCTTTAGGATCCATGAGCTCAACCATGGATGTCGACTACCGGGAT TCTAGATTCTGCA	
OAH56	GGTACCCAAGTTGAGTATGTGGCTTCTATTGATG	
OAH57	GAGCTCTTCTGCTTAAGTTTACATAATCAGC	

proteose peptone, 10 g/liter yeast extract, 1 g/liter sodium thioglycolate) was used to grow *C. perfringens* for β-glucuronidase assays.

**Electroporation of *E. coli* and *C. perfringens*.** Standard protocols were used for electroporation of plasmids into *E. coli* strain DH10B (5). Electroporation of *C. perfringens* was done, based on a previously published method (1), as follows: cells (3 ml) were grown anaerobically overnight in BHI, resuspended, and washed twice in sucrose electroporation buffer (333 mM sucrose, 6.7 mM NaHPO<sub>4</sub> [pH 7], 1 mM MgCl<sub>2</sub>). Plasmid DNA and 400 μl of cell suspension were placed in a 4-mm cuvette on ice, and electroporation was done in an anaerobic chamber using an ECM 630 Electro Cell Manipulator (BTX, Harvard Apparatus). After electroporation, cell suspensions were incubated in BHI anaerobically at 37°C for 3 h, spread onto BHI plates with the appropriate antibiotics, and incubated anaerobically at 37°C until colony formation was observed.

**Plasmid construction.** To construct pAH2, we amplified the *bgaR*-P<sub>bgaL</sub> region from strain 13 by PCR using the primers OAH1 and OAH2 and Phusion polymerase (New England Biolabs). Adenine residues were added to the ends of the PCR product using *Taq* polymerase, and the PCR product was ligated to pGEM-T Easy (Promega) to create plasmid pAH7. The insert was sequenced to determine if any mutations had occurred during amplification. We digested pAH7 with XhoI and PstI, and the *bgaR*-P<sub>bgaL</sub> fragment was ligated to XhoI- and PstI-digested pSM240 (9) to create pAH2. Plasmid pAH1 was constructed in the same manner but was discovered to have a frameshift mutation in *bgaR* (within codon 119 out of 279), causing translation to terminate after the addition of 14

aberrant amino acids. As such, it provides a useful negative control for experiments testing plasmid *bgaR* activity. To construct pKRAH1, we designed complementary primers (OKR055 and OKR056) that, when annealed, would contain several restriction recognition sequences rarely found in the *C. perfringens* chromosome and would have ends compatible with HindIII- and PstI-digested DNA. We ligated the annealed OKR055/OKR056 primers to pAH2 digested with PstI and partially digested with HindIII to avoid cutting at a second HindIII site internal to the *bgaR* gene. To construct pKRAH-Y, the *yfp* gene in plasmid pSW4-YFP (23) was amplified using PCR with primers OAH13 and OAH14, which contain a SacI and SacII site, respectively, and ligated to SacI- and SacII-digested pKRAH1. To construct pKRAH-YB, the *yfp* gene in plasmid pSW4-YFP was amplified with primers OAH14 and OAH36, and we then amplified the *pilB* gene from strain 13 chromosomal DNA by using primers OAH37 and OAH38. Using these partially overlapping PCR products as the template, we amplified *yfp-pilB* using OAH14 and OAH38 and ligated the product into pGEM-T Easy as described above, creating plasmid pAH11. pAH11 and pKRAH1 were then digested with SacII and SacI and ligated together to form pKRAH-YB.

**Mutation of the *bglR* and *bgaR* genes in strain 13.** An 890-bp fragment internal to *bglR* was amplified from strain 13 by using the primers OAH5 and OAH6 and Phusion polymerase. Adenine residues were added to the ends of the PCR product by using *Taq* polymerase, and the PCR product was ligated into pGEM-T Easy to create plasmid pAH3. Plasmid pAH3 was digested with KpnI

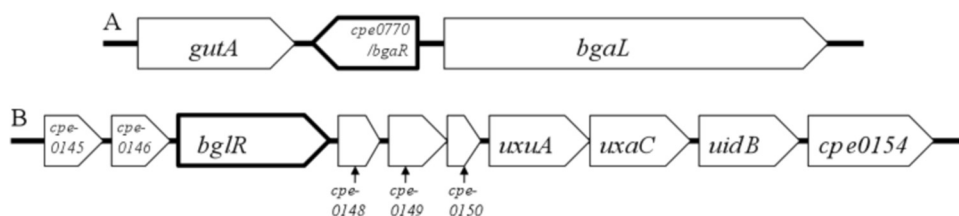


FIG. 1. (A) Gene order surrounding the *bgaR* (CPE0770) gene, an AraC family transcriptional regulator, in *C. perfringens* strain 13 (25). *gutA* encodes a probable sugar transport protein, and *bgaL* encodes a  $\beta$ -galactosidase. (B) Genes in the operon that includes *bglR*, a probable  $\beta$ -glucuronidase in strain 13. Gene CPE0145 is annotated as encoding a hypothetical protein, CPE0146 a 2-keto-3-deoxygluconate kinase, CPE0148 a putative transcriptional regulator, CPE0149 a putative oxidoreductase, CPE0150 a 2-dehydro-3-deoxyphosphogluconate/4-hydroxy-2-oxoglutarate aldolase, *uxuA* a D-mannanate dehydrogenase, *uxuC* a glucuronate isomerase, *uidB* a glucuronide permease, and CPE0154 a putative beta-hexosaminidase A.

and *SacI* to release the *bglR* gene fragment, which was ligated to pSM300, forming pAH4. Plasmid pSM300 is a suicide plasmid carrying an *ermBP* resistance gene (9). Plasmid pAH4 was transformed into *C. perfringens* by electroporation and erythromycin-resistant (Erm<sup>r</sup>) recombinants selected using BHI plates with erythromycin. An Erm<sup>r</sup> transformant (strain AH1) was confirmed to have an insertion in the *bglR* gene by PCR amplification using primers that annealed within pAH4 and to an area of the chromosome outside the homologous region used for integration. Strain 13 chromosomal DNA was screened as a negative control. PCR using primers designed to amplify the *bglR* wild-type gene produced a very faint band in the *bglR* mutant strain AH1, which did not disappear after several days of growth in BHI, which suggested that there was a very small percentage of wild-type bacteria present, probably due to infrequent recircularization of the integrated plasmid via the regions of homology. A mutation was introduced into the *bgaR* gene in the same way, except a 389-bp fragment of *bgaR* was amplified from strain 13 with primers OAH56 and OAH57, cloned into PGEM-T Easy, creating pAH5, and then ligated into pSM300, creating pAH6. The mutant (strain AH2) was constructed and screened as described above for strain AH1.

**$\beta$ -Glucuronidase assays and  $\beta$ -galactosidase assays.**  $\beta$ -Glucuronidase assays were performed on mid-log-phase cells, and specific activity was calculated according to the protocol previously described by Melville et al. (16).  $\beta$ -Galactosidase assays were performed the same way, except that *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) was used as the substrate. For the timed induction assays, the cells were subcultured into 500 ml of prewarmed PY without inducer and grown to mid-log phase. The inducer was then added, and samples were removed at different time points and placed onto dry ice. The rest of the assay followed the same protocol as that described by Melville et al. (16).

**Fluorescence microscopy.** For localization of YFP, 2% agarose pads with BHI, 0.1 mM lactose, and 20  $\mu$ g/ml chloramphenicol were placed on slides as previously described (27). The slides were placed into a Coy anaerobic chamber, and bacteria were spread on them. After 2 h of growth, the slides were removed from the chamber and exposed to aerobic conditions for at least 20 min, and then a coverslip was placed on top and sealed with nail polish. Cells were viewed on an Olympus IX71 microscope using the Applied Precision deconvolution SoftWorx program. For localization of fluorescent proteins, images of >270 individual cells were examined.

## RESULTS

**Construction of a lactose-inducible promoter plasmid.** We analyzed the genome of strain 13 for putative regulators of carbohydrate metabolism that were oriented in the opposite direction from the genes they regulated, similar to *araC* and the *araBAD* operon in *E. coli* (8). An essential enzyme in most lactose metabolic pathways is  $\beta$ -galactosidase (19). *C. perfringens* strain 13 possesses four genes that have been annotated to encode  $\beta$ -galactosidases (assigned gene designation): CPE0167 (*pbg*), CPE0771 (*bgaL*), CPE0831 (also *bgaL*), and CPE1266 (none) (25). Of these  $\beta$ -galactosidase-encoding genes, only CPE0771 (*bgaL*) had a divergently oriented transcriptional regulator associated with it, CPE0770 (Fig. 1A). CPE0770 was annotated as encoding an AraC family transcrip-

tional regulator (25). Because CPE0770 regulates *bgaL* in a lactose-dependent manner (see below), we renamed it *bgaR* (i.e., regulator of *bgaL*). Also, because there are two genes named *bgaL* in strain 13, we recommend that CPE0771 and CPE0831 be renamed *bgaL* and *bgaM*, respectively. Upstream and divergent to *bgaR* is a gene named *gutA* (Fig. 1A), which was annotated as a probable sugar transport protein (25) and may function as a transporter of lactose. The *gutA*, *bgaR*, and *bgaL* genes and their synteny are highly conserved in all nine *C. perfringens* strains with completely sequenced (18, 25) and partially sequenced (NCBI database) genomes.

The entire *bgaR* gene and the putative promoter and N-terminal region of *bgaL* (*bgaR*-P<sub>*bgaL*</sub>) were amplified by PCR and ligated into plasmid pSM240 to create plasmid pAH2 (Fig. 2). This placed the *bgaL* promoter immediately upstream of a *cpe-gusA* reporter gene fusion present in pSM240 (Fig. 2), which consists of the *cpe* ribosomal binding site and the first 13 codons fused in frame to the entire *gusA* gene of *E. coli* (16). The *cpe* gene from *C. perfringens* strain SM101 encodes the enterotoxin (CPE) responsible for acute food poisoning and has a ribosome binding site that likely provides a high level of translation of the protein since it is expressed at very high levels in strain SM101 (16). The *E. coli gusA* gene, encoding a  $\beta$ -glucuronidase (12), has been used successfully as a reporter in *C. perfringens* (16, 32), *Clostridium acetobutylicum* (6), *Clostridium beijerinckii* (6), and *Clostridium difficile* (15).

***bgaR*-P<sub>*bgaL*</sub> expression in three pathogenic strains of *C. perfringens*.** We transformed plasmid pAH2 into three different strains of *C. perfringens* and assayed  $\beta$ -glucuronidase activity at increasing concentrations of lactose (Fig. 3). Strain SM101, an acute food poisoning-associated strain, has a low endogenous  $\beta$ -glucuronidase activity of ~4 to 8 U (16). We observed about an 80-fold increase in expression at maximum induction (10 mM lactose) and just 2-fold-increased expression above background levels in the absence of inducer (Fig. 3A). JGS4143, a strain isolated from an outbreak of chicken necrotic enteritis (G. Songer, personal communication), had 50 U of endogenous  $\beta$ -glucuronidase activity but showed lactose-inducible expression with pAH2 up to 10 times that of the background level (Fig. 3B). Strain 13, the source of *bgaR*-P<sub>*bgaL*</sub>, also has significant (45 to 70 U) background  $\beta$ -glucuronidase activity (Fig. 3C) (17), but with pAH1 present, we could detect about 4-fold-increased  $\beta$ -glucuronidase activity as the lactose concentration increased (Fig. 3C).

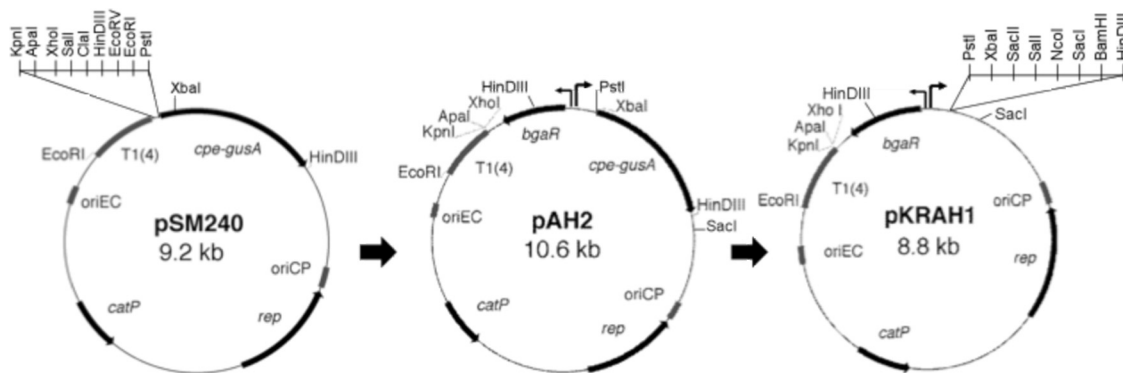


FIG. 2. Relevant features of plasmids described in this report. Plasmid pSM240 was briefly described previously (9). T1(4) denotes four tandem terminators (26).

***bglR* in strain 13 encodes a  $\beta$ -glucuronidase.** Strain 13 is the most commonly used strain in *C. perfringens* pathogenesis and gene regulation studies (20, 21). Due to endogenous  $\beta$ -glucuronidase activity (Fig. 3C), it is difficult to gauge the precise levels of induction from the *bgaR*- $P_{bgaL}$  construct. Therefore, we located a gene that was annotated to encode a putative  $\beta$ -glucuronidase, *bglR*, in strain 13 (25). The *bglR* gene is the second gene in a nine-gene region that likely forms an operon (Fig. 1B). Based on comparison to the *E. coli* genes involved in glucuronide metabolism (19), the *C. perfringens* strain 13

operon appears to encode enzymes involved in metabolizing  $\beta$ -glucuronides. Using NCBI BLAST analyses with genes from strain 13, the genes associated with  $\beta$ -glucuronide metabolism are conserved in *C. perfringens* strains F4969, JGS1721, JGS1495, JGS1987, and NCTC 8239 but absent in strains ATCC 13124, SM101, and ATCC 3626.

We introduced a mutation into the *bglR* gene of strain 13 via homologous recombination of a nonreplicating plasmid (see Materials and Methods). The mutant strain, AH1, exhibited an 89% decrease in the  $\beta$ -glucuronidase activity at 0 mM lactose

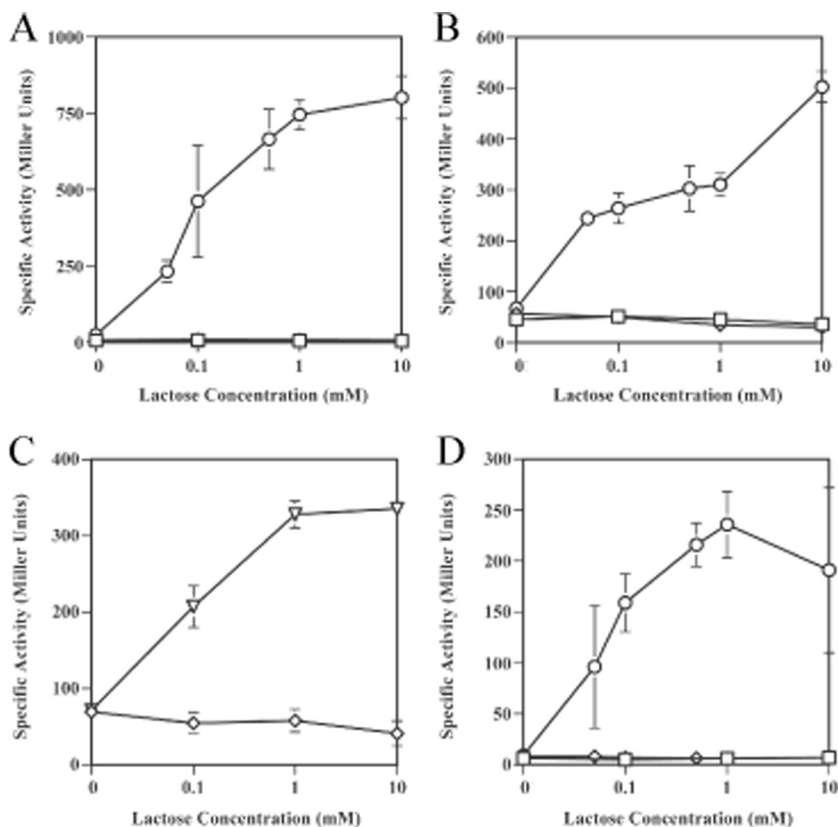


FIG. 3.  $\beta$ -Glucuronidase activity in *C. perfringens* at different concentrations of lactose. (A) Strain SM101; (B) strain JGS4143; (C) strain 13; (D) strain AH1. Strains containing no plasmid (squares), pSM240 (diamonds), pAH2 (circles), and pAH1 (triangles). Except for the sample at 0 mM lactose, lactose concentrations are shown on a log scale.



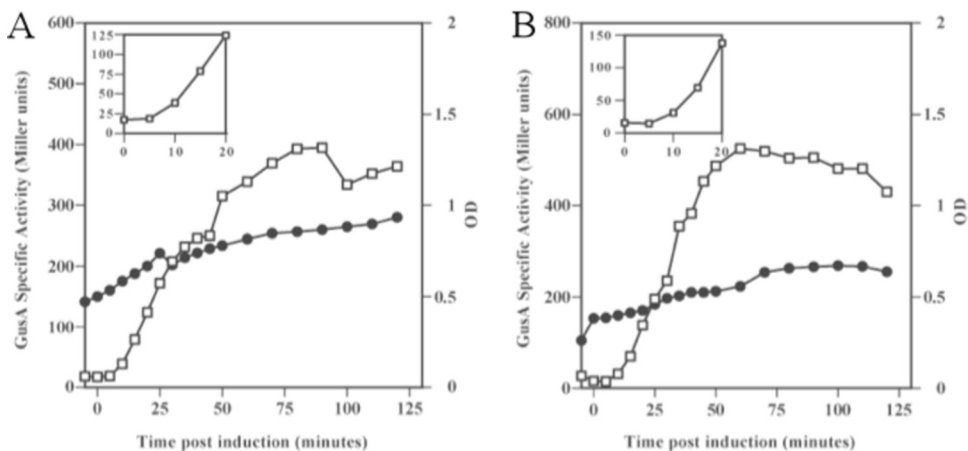


FIG. 4. Induction of  $\beta$ -glucuronidase activity (squares) at 1 mM lactose with strain AH1 (*bglR* mutant) (A) and SM101 (B) containing pAH2. OD (optical density at 600 nm) is shown as filled circles. Each chart shows a representative sample from two independent trials. The insets show an enlarged image of the first 20 min after induction.

(Fig. 3D), suggesting that *bglR* encoded an enzyme responsible for the majority of  $\beta$ -glucuronidase activity. In strain AH1, pAH2 produced lower activity in the absence of inducer than did pAH2 in strain SM101 (compare Fig. 3D to A).

Also, we tried substituting a 10 mM concentration of the gratuitous inducer isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) in place of lactose as an inducer of  $P_{bgaL}$  but did not observe an increase in activity (data not shown), suggesting that IPTG either is not an inducer or is not transported into the cell.

**Expression from *bgaR*- $P_{bgaL}$  begins 5 to 10 min after the addition of an inducer.** To determine the kinetics of induction of the *bgaR*- $P_{bgaL}$  construct, we added 1 mM lactose to two strains, AH1(pAH2) and SM101(pAH2), in mid-log phase and measured the induction of activity over time (Fig. 4). The  $\beta$ -glucuronidase activity began to increase between 5 and 10 min after the addition of lactose and reached maximum levels between 1 to 2 h after lactose was added (Fig. 4).

**Glucose has no significant effects on induction from *bgaR*- $P_{bgaL}$ .** We performed all our assays in PY, a low-carbohydrate medium, as carbohydrate-inducible promoters in *E. coli* have exhibited repression in the presence of glucose (8). To determine if glucose has an effect on *bgaR*- $P_{bgaL}$  expression, we tested the effects of adding 10 or 110 mM glucose (the amount present in PGY media [16]) to strains AH1(pAH2) and SM101(pAH2). Neither 110 mM glucose nor 10 mM glucose had a significant effect on *bgaR*- $P_{bgaL}$  expression (data not shown).

***bgaR* is the activator responsible for the lactose-induced activity.** It was possible that the results described above could be due to the activity of a transcriptional regulator other than BgaR on the *bgaL* promoter. To ensure that BgaR was the protein responsible for regulating the *bgaL* promoter, we introduced a mutation into the chromosomal copy of the *bgaR* gene (see Materials and Methods) to create strain AH2. We then individually transformed pAH2 and pAH1 into this strain. Plasmid pAH1 has the same overall structure as pAH2, but the *bgaR* gene contains a frameshift mutation at residue 119 (out of 279) that leads to a prematurely terminated protein of 133

residues in length that is inactive. The  $\beta$ -glucuronidase expression in strain AH2(pAH2) upon addition of lactose was similar to that seen with strain 13(pAH1) (Fig. 5). However, strain AH2(pAH1), which lacks any wild-type copy of the *bgaR* gene, showed a complete lack of induction in the presence of lactose, indicating that BgaR is responsible for lactose-mediated induction. Since the absence of BgaR leads to loss of induction (Fig. 5), it is likely that BgaR functions as a transcriptional activator and not a repressor in response to lactose.

The *bgaR* mutant strain was examined to see if the mutation affected the total amount of the  $\beta$ -galactosidase activity, measured by hydrolysis of ONPG, in the cell. The level of  $\beta$ -galactosidase activity was relatively low in the wild-type strain (10 to 14 U) and showed limited induction in the presence of lactose (Fig. 6). However, there was a significantly reduced level of enzyme activity in the *bgaR* mutant strain at each concentration of lactose except 0.1 mM, and the limited amount of lactose-dependent induction was lost (Fig. 6). Because the

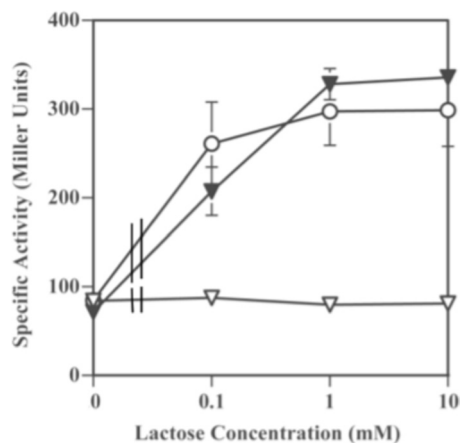


FIG. 5.  $\beta$ -Glucuronidase activity in response to lactose concentration. Strain AH2 (open symbols) with pAH1 (triangles) and pAH2 (circles). For comparison, strain 13 with pAH1 (same data as that shown in Fig. 3C) is shown by filled triangles.

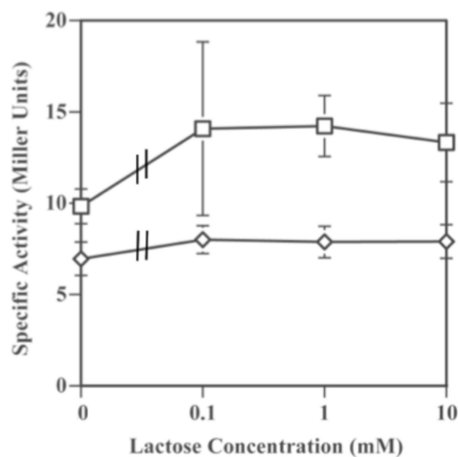


FIG. 6.  $\beta$ -Galactosidase activity in response to lactose concentration. Strain 13 (squares) and strain AH2 (*bgaR* mutant) (diamonds).

activity of ONPG hydrolysis was relatively low, we performed a control where the *E. coli lacZ* gene, encoding  $\beta$ -galactosidase, under the control of the *cpe* gene promoter from strain SM101, was placed on a plasmid which was transformed into strain 13. This strain exhibited a significant increase in ONPG hydrolysis (data not shown), indicating that there were no limitations to measuring ONPG hydrolysis in our assays.

**Replacement of the *cpe-gusA* reporter with a multiple cloning site allows controlled expression of desired proteins.** After determining that pAH2 works well for controlled induction, we replaced the *cpe-gusA* gene fusion with a multiple cloning site containing restriction enzyme recognition sites that are rarely found in *C. perfringens*. The resulting plasmid, pKRAH1 (Fig. 2), allows lactose-induced controlled expression of any gene desired.

**YFP-PilB localizes to the pole in *C. perfringens* strain 13 but not in a *pilC* mutant strain.** We have previously shown that type IV pili (TFP) have a role in *C. perfringens* gliding motility (30). One of the gene clusters involved in TFP motility was an operon containing the genes *pilB*, *pilC*, *CPE1842*, and *CPE1841*. The *pilB* gene product is likely an ATPase responsible for assembly and extension of TFP (30). The *pilC* gene product is a membrane protein that we showed was necessary for TFP gliding motility in *C. perfringens* by mutagenesis of the gene (30). PilC homologs have been shown to aid in localization of PilB to the poles of cells in Gram-negative bacteria with TFP, including *Pseudomonas aeruginosa* (2). To determine whether PilC performed the same in the Gram-positive *C. perfringens*, we inserted genes encoding YFP and a YFP-PilB fusion protein, in which YFP was attached to the N terminus of PilB, into pKRAH1. In medium without lactose, there was no visible fluorescence (data not shown). While expression of the *yfp* gene alone led to diffusion of the YFP protein throughout the cytoplasm of the bacteria (Fig. 7A), the YFP-PilB protein localized to the pole in 73% of the cells (Fig. 7B and C). In strain SM126, the previously constructed nonmotile *pilC* mutant derived from strain 13 (30), the fluorescence was still punctate, but only 42% was on the cell poles, and the fluorescent signals were less intense than the polar fluorescence in strain 13 (Fig. 7D). These results suggest that the PilC protein

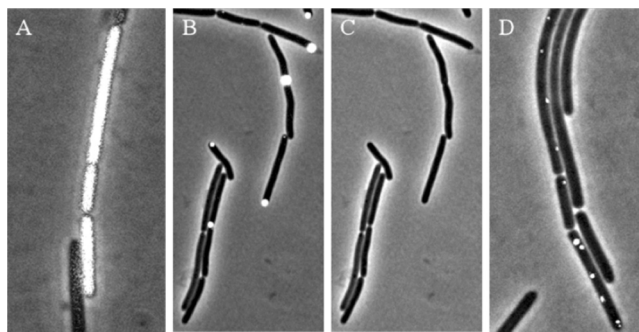


FIG. 7. Cellular localization of YFP and the YFP-PilB fusion protein. Strains were grown on agarose pads with BHI and 0.1 mM lactose before images of cells were collected with phase-contrast microscopy and fluorescence microscopy (where indicated). (A) Strain 13 with pKRAH-Y, showing YFP fluorescence (white coloration in cells) distributed evenly throughout the cell. (B) Strain 13 with pKRAH-YB, in which the fluorescence (white spots) is distributed mostly to the poles. (C) Same image as that in panel B but with phase contrast only to distinguish the location of cell poles. (D) Strain SM126, a *pilC* mutant derived from strain 13, with pKRAH-YB. Fluorescence intensity levels were adjusted to the same minimum and maximum levels for the images in panels B and D so they could be directly compared to each other.

is at least partly responsible for polar localization of PilB in *C. perfringens*.

## DISCUSSION

Two gaps in the genetic tools available for *C. perfringens* were the absence of a vector in which promoters from genes of interest could be studied in the native *C. perfringens* host and a readily usable vector for highly controlled gene expression. Plasmid pSM240, which fulfills the first criterion, was briefly described previously, where it was used to analyze the expression patterns of promoters from the *sigK* and *spoIIIGA* genes from strain SM101 (9), but a detailed description of its utility was not provided. Because it has a polylinker between a region containing four tandem terminators and the *cpe-gusA* reporter gene fusion (Fig. 2), it can be used to study the regulation of almost any promoter found in *C. perfringens*. The effectiveness of the terminators can be seen in Fig. 3A, B, and D, where the strains containing pSM240 show almost no additional  $\beta$ -glucuronidase activity over that of the wild-type strain lacking any plasmids. The *cpe-gusA* reporter in pSM240 is more useful in a *C. perfringens* strain 13 background if the *bglR* mutant strain, AH1, is used as the host. Since the endogenous  $\beta$ -glucuronidase activity was reduced from  $\sim 70$  U to  $\sim 8$  U in strain AH1, it will make possible the analysis of all but weak promoters.

We used a regulator of a putative  $\beta$ -galactosidase-encoding gene and its adjacent promoter as the basis for constructing a vector to fulfill the second need for highly controlled gene expression. We deliberately searched for sugar metabolism genes with a divergent regulator and promoter, because this orientation is more likely to avoid read-through transcription from an upstream gene in the same orientation. This has been clearly demonstrated with the pBAD family of expression vectors developed by Guzman and coworkers (8). In the case of the pBAD plasmids, the promoter could be repressed by the

TABLE 2. Characteristics of four putative  $\beta$ -galactosidases in *C. perfringens* strain 13

$\beta$ -Galactosidase	% sequence similarity to:				Other bacteria with homology	Distinguishing characteristic(s)
	BgaL (CPE0771)	BgaM (CPE0831)	Pbg (CPE0167)	CPE1266		
BgaL (CPE0771)		17.3	10.4	15	<i>Firmicutes</i>	Homologous proteins often annotated as glycoside hydrolases
BgaM (CPE0831)	17.3		10.2	24.2	<i>Agrobacterium-Sinorhizobium</i>	None
Pbg (CPE0167)	10.4	10.2		10.2	<i>Firmicutes</i>	Transcription shown to be induced by lactose (13) and cleaved X-Gal when cloned into an <i>E. coli</i> plasmid and activated by ONPG (24)
CPE1266	15	24.2	10.2		<i>Bifidobacterium</i> and pathogenic members of the <i>Firmicutes</i>	Potential secretion signal sequence at the N terminus; homologous proteins are surface anchored and contribute to avoidance of phagocytosis by inhibiting complement deposition on the surface of the bacterium (4)

addition of glucose to the medium, giving a larger range of regulation (8), but the *bgaR-P<sub>bgaL</sub>* in pAH2 was not. Although this may limit the range of transcriptional regulation, it can also be viewed as a positive feature since *C. perfringens* can be grown in rich medium with high levels of glucose without repression, which would be valuable for obtaining high levels of synthesis of a target protein for purification or other uses.

The *bgaR-P<sub>bgaL</sub>* construct in pAH2 was regulated efficiently by lactose in three different strains of *C. perfringens* that have three different pathogenesis profiles, gas gangrene (strain 13), acute food poisoning in humans (strain SM101), and necrotic enteritis in chickens (strain JGS4143) (Fig. 3). Since the *gutA-bgaR-bgaL* operon was found in all sequenced strains of *C. perfringens*, with GutA being the putative lactose permease, it suggests that this construct can be utilized in virtually any strain in this species. Although not tested yet, *bgaR-P<sub>bgaL</sub>* may also provide regulated expression in other *Clostridium* species as long as they can transport lactose into the cell.

Sequence alignment of the four potential  $\beta$ -galactosidase proteins in strain 13 reveals a remarkable degree of divergence in the sequences, since the pair with the highest level of similarity, BgaM and CPE1266, exhibit only 24.2% sequence identity (Table 2). A search for homologs of the four  $\beta$ -galactosidase enzymes indicates that they are found in all strains of *C. perfringens* with sequenced genomes (data not shown). Distinguishing characteristics of the  $\beta$ -galactosidases are listed in Table 2. For still unknown reasons, it appears that *C. perfringens* has evolved multiple mechanisms for facilitating the metabolism of lactose.

Despite strain 13 having multiple putative  $\beta$ -galactosidase enzymes, we detected a relatively low level of enzyme activity (10 to 14 U) in strain 13 by using ONPG as the substrate, and this activity was only slightly affected by the presence of lactose (Fig. 6). Loss of *bgaR* function resulted in decreased activity, but 60% of the activity remained (Fig. 6). Since we have observed an 80-fold induction of the *bgaL* promoter by adding lactose (Fig. 3), it seems likely that the BgaL enzyme does not hydrolyze ONPG as a primary enzymatic activity.

Kobayashi et al. suggested that CPE0166 (ORF54) was a lactose transporter, likely a permease (13). The *gutA* gene

product, the putative lactose permease divergent to the *bgaR* gene (Fig. 1A), has little sequence similarity with CPE0166 and was likely given the *gutA* designation because it has similarity to glucuronide transport proteins (25). No obvious transporter was found nearby either the *bgaM* gene or *CPE1266*, which encode the other two putative  $\beta$ -galactosidases in strain 13 (data not shown).

Another useful outcome of this study was the confirmation of the gene assignment that *bglR* encoded a  $\beta$ -glucuronidase in strain 13. This was done by mutagenesis of the *bglR* gene and by showing that the mutant had a significant decrease in  $\beta$ -glucuronidase activity (Fig. 3D). However, the method used for mutagenesis, insertion of a nonreplicating plasmid by homologous recombination, is likely to have polar effects on downstream genes in the locus, so we cannot exclude the possibility that one of these genes is also responsible for this phenotype. The remaining 11% of  $\beta$ -glucuronidase activity in the *bglR* mutant strain may be due to residual activity from another  $\beta$ -glucuronidase enzyme that cannot be detected by sequence homology to known  $\beta$ -glucuronidases or nonspecific activity of another hexuronidase. Strain SM101 lacks the *bglR* gene yet has low but detectable  $\beta$ -glucuronidase activity of 4 to 8 U (Fig. 3A), suggesting that other enzymes with this activity exist in *C. perfringens*.

To demonstrate the utility of the expression system, we used the pKRAH1-based vector to express a gene encoding a YFP-PilB protein fusion and showed that polar localization of PilB is due, at least in part, to the membrane-bound protein PilC (Fig. 7). This is similar to results seen in the Gram-negative bacterium *P. aeruginosa* and suggests that PilC functions as the membrane-bound partner for the PilB protein in *C. perfringens*. Expression of heterologous proteins, including the green fluorescent protein (GFP) family, in *C. perfringens* should provide the means to make significant discoveries related to gene and protein functions in this bacterium.

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