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Development of an IPTG Inducible Expression Vector Adapted for *Bacteroides fragilis*

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Abstract

The genus *Bacteroides* are gram-negative, obligate anaerobes indigenous to the gastrointestinal tract of humans and animals. The *Bacteroides* and other members of the *Bacteroidetes* phylum have diverged from the *Proteobacteria*. These organisms evolved a unique promoter structure for the initiation of transcription, hence common genetic tools are of limited use in the *Bacteroides*. An expression vector that can control gene expression in the *Bacteroides* was constructed by engineering the *lacO*_{1,3} repressor binding sites into the promoter of the *cfxA* β -lactamase gene. The gene for the LacI repressor was placed under control of the *Bacteroides tetQ* gene promoter for constitutive expression and inserted into the vector. Studies utilizing the xylosidase reporter gene, *Xa*, showed that the gene was induced by Isopropyl β -D-1-thiogalactopyranoside (IPTG) in a time and concentration dependent manner from 10–250 μ M over a 10–240 min time frame. The utility of the vector was demonstrated by insertion of the *Bacteroides fragilis trxA* gene into the plasmid. TrxA synthesis was monitored by Western hybridization and the results indicated that it was regulated by the presence of IPTG in the media. This is the first transcriptional regulatory system developed for the *Bacteroides* that has incorporated components from the *Proteobacteria* and demonstrates the feasibility of modifying existing genetic tools for use in these organisms.

Keywords

Bacteroides fragilis; expression vector; LacI

1. Introduction

The *Bacteroides* are Gram-negative anaerobes that inhabit the gastrointestinal tract of mammals. They are part of the unique phylum, *Bacteroidetes*, that is significantly diverged from the main *Proteobacteria* lineages and possess a number of novel biochemical properties such as the presence of membrane sphingolipids (An et al 2011; Karlsson et al 2011; Paster et al 1994; Smith et al 2006). These organisms make up a significant portion, 20–60%, of the total indigenous microflora of the colon depending on diet and other factors, and thus their contribution to human health is considerable (Eckburg et al 2005; Ley et al 2006; Wu et al 2011). As members of the normal flora they influence the development of the immune system and contribute to a wide range of physiological activities associated with the colon

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including the digestion of complex carbohydrates, energy generation in the form of short chain fatty acids, vitamin synthesis, and bile acid metabolism. In addition to the health benefits, *Bacteroides* are important opportunistic pathogens being the most frequently isolated anaerobes from infections (Finegold and George 1989). These organisms predominate in intraabdominal and pelvic infections and are the most common anaerobe isolated from blood (Comstock and Tzianabos 2000; Lassmann et al 2007).

There is considerable interest in the *Bacteroides* as model systems for the normal intestinal microflora and for endogenous opportunistic infections, both of which require genetic tools for functional analyses. One basic need is for an expression vector system that allows regulated gene expression in *Bacteroides* species. Typical systems used for prokaryotes rely on the *lac* or *ara* promoters of *Escherichia coli* and these have proven invaluable in studies for a wide range of bacterial species. However, early work showed that the *Bacteroides* are unable to express genes under control of the *lac* promoter and in fact they do not recognize the *E. coli* consensus promoter or typical gram-positive promoters (Smith et al 1992). Subsequent studies have shown that *Bacteroides* species have a unique promoter structure that is recognized by its core RNA polymerase with σ^{70} (Bayley et al 2000; Vingadassalom et al 2005). The recognition sequence, TTTG (N₁₉₋₂₁)TANNTTTG, is centered around bases -7 and -33 relative to the TIS (transcription start site). Interestingly this promoter sequence also was shown to be conserved in the *Flavobacteria*, another member of the *Bacteroidetes* phylum (Chen et al 2007).

Given this limitation in transcription initiation there are two options for the development of expression vectors. First would be to identify an inducible gene system in *Bacteroides* that could be easily manipulated, but there is a paucity of information on specific transcriptional regulatory factors in these organisms. A second approach would be to modify an existing, well proven system so that it responds to the *Bacteroides* transcriptional machinery. In this paper we have taken the *E. coli lac* system, with all its accumulated knowledge, to construct an inducible expression system that responds to Isopropyl β -D-1-thiogalactopyranoside (IPTG). The LacI repressor and the *lac* operator binding sites were engineered onto a plasmid containing the relatively strong *cfxA* promoter. Using transcriptional fusions and Western Blot techniques the system was shown to be induced by IPTG in a time and concentration dependent manner.

2. Materials and methods

2.1 Bacterial strains and growth

B. fragilis strain 638R was the *Bacteroides* host strain for all studies (Table 1). Strains were routinely grown in Difco Brain Heart Infusion Broth supplemented with hemin (0.005 g/l), NaHCO₃ (2 g/l) and cysteine (0.5g/l) [BHIS]. Antibiotics were added as needed at the following concentrations: Rifampicin (Rf, 20 μ g/ml), Gentamicin (25 μ g/ml) and Erythromycin (Em, 10 μ g/ml). Cultures were incubated in a Coy anaerobic chamber with an atmosphere of 80% N₂ 10% CO₂ and 10% H₂ at 37°C. *E. coli* EC100 was used for routine DNA manipulations and cloning. Plasmids constructed in *E. coli* were mobilized into *Bacteroides* strains by triparental mating using RK231 as a conjugal helper plasmid (Bacic et al 2005). Cultures were grown Luria-Bertani (LB) medium with appropriate antibiotics as described in the text: Spectinomycin (Sp, 60 μ g/ml) and Kanamycin (Km, 25 μ g/ml).

2.2. Construction of pFD1146

Shuttle vector, pFD288, was the base plasmid used for construction of pFD1146. First, the constitutive *cfxA* promoter was amplified from pFD351 using a primer, Cfx-lacO1-R, that incorporated the *lacO*₁ operator site 1 bp downstream of the *cfxA* TIS and a second primer, Cfx-F, about 340 bp upstream of the promoter (Table 2). This fragment was cloned into

SphI/SalI digested pFD288. This construct was the template for primers Cfx-lacO3-F and Cfx-R which incorporated the *lacO*₃ site, 42 bp upstream from the –33 region of the *cfxA* promoter and encompassed an EcoRV site downstream of the of the promoter region. The resulting amplified fragment containing the *lacO*₃, *cfxA* promoter, and the *lacO*₁ site was digested with SphI + EcoRV and cloned into similarly digested pFD288 to form pFD1140. Next the *lacI* gene was amplified from pSRKKm using primers Laci-F and Laci-R, and then inserted into a plasmid downstream of the *tetQ* promoter to form pFD1142. Finally the *tetQ*, *lacI* transcriptional fusion was amplified from pFD1142 using primers I-tet-F and I-tet-R, digested with EcoRI+SmaI and then ligated into EcoRI + EcoRV digested pFD1140 to form the final product pFD1146. The nucleotide sequence of this plasmid, GenBank Accession, JQ776640, was determined by deduction from the previously reported sequences of pFD288 and the other components, and it was confirmed by sequence analysis of the newly modified regulatory regions between nucleotides 7600 and 9400.

The xylosidase reporter gene, *XA*, originally obtained from pXA1 (Whitehead 1997) on a BamHI fragment was ligated into the expression vector to form pFD1147. Another construct, pFD1153, contained the *B. fragilis trxA* gene (Reott et al 2009) with a c-Myc tag at the C-terminus cloned into pFD1146.

2.3. Xylosidase transcriptional fusion assay

To determine the activity of xylosidase transcriptional fusions *B. fragilis* cells containing the appropriate plasmid were grown in BHIS media to mid-logarithmic phase (A550 0.3–0.6) and were harvested by centrifugation. The cells were suspended in 1.35 ml 50mM sodium phosphate buffer (pH 6.8) and the cell solution was divided to three equal aliquots. Fifty μ l CTAB solution (0.05% hexadecyltrimethylammonium bromide + 10mM dithiothreitol in 50mM sodium phosphate buffer) was added to each tube, mixed and incubated at 37°C. Next 10 μ l 100mM p-nitrophenyl-D-xylopyranoside in dimethyl sulfoxide was added to each reaction, and incubated at 37°C for 15 min. The reaction was stopped by the addition of 50 μ l of 2% Na₂CO₃ and cells were removed by centrifugation. The absorbance of the supernatant at 405nm was recorded. Activity units were determined by the formula $[A_{405}/(T \times V \times A_{550})] \times 1000$; where T is equal to time of assay in min, and V is the volume of cells calculated to the original culture.

2.4. Western hybridizations

Samples for Western blots were obtained from cultures grown with IPTG induction as described in the text. Cultures were centrifuged and stored at –80°C until SDS-PAGE was performed as described previously (Laemmli 1970). Samples were adjusted to equal amounts of protein and then 16–20 μ g of each was added to loading buffer containing running dye in a total volume of 20 μ l, boiled for 10 min, allowed to quickly cool on ice, and then loaded on a 12% SDS-PAGE gel. Proteins then were transferred to a PVDF membrane, probed with primary antibody, then labeled with an alkaline phosphatase secondary antibody, and bands were visualized using chromogenic detection with nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolylphosphate. Digital densitometric analysis of photographic images was performed using ImageQuant software (Amersham Bioscience) to estimate relative band intensity. Densitometric measurements from at least 2 independent gels were obtained. Experiments measuring the c-Myc tagged TrxA utilized a monoclonal c-Myc primary antibody (Sigma) and anti-mouse secondary antibody. The LacI protein was identified with rabbit anti-LacI (Lifespan Bioscience, Seattle) and anti-rabbit IgG secondary.

3. Results and discussion

3.1. Strategy and construction of a *Bacteroides* expression vector

We previously constructed a constitutive *Bacteroides* expression vector based on the outward firing promoter of IS4351 (Smith, Rogers, and McKee 1992). This plasmid has been used extensively for complementation experiments but it has obvious drawbacks for experiments where more precise regulation of gene expression is desired. In this regard the starch/maltose inducible *osuA* promoter has been cloned into vector pFD1045 and used to control *B. fragilis* gene expression in a more responsive manner (Lobo et al 2011; Spence et al 2006). Although this system has allowed for some “user” controlled regulation of gene expression, the *osuA* promoter is subject to induction by aerobic stress and by growth phase so these factors have limited its utility. To overcome some of these shortcomings an expression system was designed around the *lacI* repressor which would be IPTG inducible. This avoids some of the problems surrounding the use of native *Bacteroides* systems where there are still relatively few details for any substrate inducible gene systems. Further, IPTG is apparently freely permeable across cell membranes so the lack of suitable transport systems is not a factor for induction of gene expression.

Several modifications were necessary to adapt the *lacI* system to the *Bacteroides* transcriptional machinery. The first consideration was to replace the *lacZ* promoter with one recognized by the *Bacteroides* RNA polymerase while at the same time retaining the *lacO* operator sites. This was accomplished by cloning the constitutive *cfxA* promoter from Tn4555 into the shuttle vector pFD288. Two sequential cloning steps were used with primer pairs that contained either the *lacO₁* or *lacO₃* repressor binding sites. Previous work has shown that the spacing between the *lacO* operators was critical for maximal LacI repression (Oehler et al 1990). The construction strategy used here maintained that optimal spacing for the *lacO₃* binding site with respect to the -33 and the *lacO₁* site with respect to the TIS (Fig. 1). The *lacO₂* site was not incorporated into this vector since its location in the 5′ end of the *lacZ* gene would not be practical for routine use in an expression vector.

The second modification needed was to provide for adequate expression of the cloned LacI structural gene. A 504 bp fragment containing the *tetQ* promoter from CTn341 was utilized for constitutive transcriptional activation of *lacI*. The cloned promoter fragment did not include the tetracycline attenuation leader sequence located between the TIS and the *tetQ* structural gene, thus LacI was not subject to tetracycline induction (Wang et al 2005). Another concern was to allow for optimal translation of the gene product. Although the *B. fragilis* ribosome binding site (RBS) has not been specifically determined, the complement of the 16S rRNA 3′ end is, AGAAAGGAG. This is not a good match with the native *lacI* RBS thus the *B. fragilis* *ahpC* gene RBS was inserted adjacent to the translation start site (Fig. 1, and supplemental data). The expression of LacI in *B. fragilis* was examined by Western hybridization analyses. As shown in Fig. 2, LacI was produced from the pFD1142 construct in 638R and readily detected as a protein band identical in size to that produced by pSRKKm in *E. coli*. There were some cross reacting proteins in 638R but these did not interfere with identification of the LacI product at about 40 kDa.

The final construct, pFD1146, was produced by insertion of the *tetQ/lacI* fusion into the plasmid containing the *cfxA+lacO_{1,3}* promoter (Fig. 3). Western hybridization showed that LacI was expressed to the same levels as shown above for pFD1142 (data not shown). The possible toxicity of LacI was gauged by growth curve studies. The results in Fig. 4 compare pFD1140 (no LacI), pFD1146 (LacI), pFD1147 (LacI + xylosidase reporter gene) to the wild type strain with no plasmids. The results show that each of the plasmid bearing strains had a slight growth defect compared to the parent strain but this was not associated with the presence of the *lacI* gene or the reporter gene. This decreased growth rate was similar to

what we have observed with other *ermF* containing plasmids in the presence of erythromycin (data not shown).

3.2. IPTG induction of the xylosidase reporter gene

The expression system was tested using the xylosidase reporter gene, *XA*, originally isolated from *Bacteroides ovatus* (Whitehead 1997). Cells containing pFD1147 were grown to mid-logarithmic phase and then IPTG from .001 – 1.0 mM was added to test induction. After two h incubation cells were harvested and xylosidase activity was measured (Fig. 5A). The promoter was not significantly induced at the lowest concentrations of IPTG but the addition of 0.05 mM resulted in an increase of about 5-fold over the control. Further induction was noted up to about 0.25 mM IPTG above which activity seemed to level off. Similarly the response of the promoter to time following induction was examined at a constant IPTG concentration of 1 mM IPTG (Fig. 5B). After about 10 min of IPTG induction xylosidase activity was observed and continued to increase in proportion to the length of time following addition. The xylosidase activity appeared to peak at about 4 h and remained high for the course of the experiment.

Induction of the reporter gene also was tested using lactose. Cells containing pFD1147 were grown to mid-logarithmic phase and then split. One half received 10 mM lactose and both were incubated for an additional 2.5 h. Analysis of xylosidase activity showed no differences between the lactose treated or untreated cultures. Although *B. fragilis* contains more than 10 genes annotated as β -galactosidases there was no obvious induction of the *cfxA* promoter suggesting that allolactose is not produced in these cells under the conditions tested.

Previous studies had shown that exposure to air induced the expression of many carbohydrate utilization systems but cultures shaken in air for an hour showed no increase in xylosidase [(Sund et al 2008) and data not show].

3.3. Regulated expression of a *B. fragilis* thioredoxin, TrxA

The pFD1146 expression system was finally tested using a native *B. fragilis* gene, *trxA*, that was modified to contain the c-Myc tag at the 3' end of the gene. As shown in Western hybridization results, Fig. 6A, there was a good correlation between the length of time following induction with 1 mM IPTG and the amount of TrxA produced. The results were similar to the reporter gene assays with near maximum induction by about 4 h. Analysis of the Western blots by densitometry showed that there was some additional TrxA produced out to at least 6 h (data not shown). The dependence of TrxA induction on the concentration of IPTG was maximal at 0.5 mM IPTG and decreased somewhat at 2 mM (Fig. 6B). This result was similar to the analyses with the XA reporter gene.

3.4. Summary and conclusions

The results reported here describe the construction of a *Bacteroides* expression vector that uses the LacI repressor to control transcription from the *cfxA* promoter in an inducer dependent manner over a 7–10-fold range of activity. This range of activity is not as great as that seen in many vectors in *Proteobacteria* systems, therefore this might be an area of future development for the system. For example it should be possible to replace the *cfxA* promoter with one of the strong *Bacteroides* 16S rRNA promoters which exhibit exceptionally high levels of transcriptional activity (Mastropalol et al 2009). Such a system would be useful for the production of *Bacteroides* proteins such as cell surface proteases or secreted enzymes that are not processed properly in typical *E. coli* host vector system (Barkocy-Gallagher et al 1999; Rasmussen et al 1991).

The expression system was reasonably effective in the repression of transcription from the *cfxA* promoter but in the absence of IPTG there still was a low level of background activity. This was true in both the xylosidase assays and the TrxA Western Blots so the system may not be useful for cloning of toxic products. This leaky expression might be caused by insufficient LacI so it might be helpful to place the *lacI* gene under control of a stronger constitutive promoter. Alternatively it might be useful to modify the codon usage of *lacI* to one that is more favorable to the *Bacteroides* translation system. These organisms have a distinct array of tRNA's that are rarely used in *E. coli* and the converse also is true.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

An IPTG inducible expression vector was designed for *Bacteroides fragilis*

The expression vector is a hybrid of the operators lacO_{1,3} from *E. coli* lacZ and the cfxA promoter from *Bacteroides*

The vector was tested with a xylosidase reporter gene and with a native *B. fragilis* gene by Western hybridization.

Fig. 1.

Nucleotide sequence and features of the *B. fragilis* *cfxA* promoter with incorporated *lacO* binding sites in pFD1146. The *cfxA* and *tetQ* promoter regions and *lacI* coding sequence are shown with the block arrows and the relative locations of the *lacO* operators are indicated. The map is drawn to scale. The nucleotide sequence shows the *cfxA* promoter with the precise location of the -7 and -33 sites relative to the *lacO_{1,3}* repressor binding sites. In addition, the unique restriction sites suitable for cloning a gene of interest are also shown.

Fig. 2.

Western hybridization showing the production of LacI from pFD1142 in *B. fragilis*. (A) Western hybridization with LacI antibody; (B) the 0.8% SDS-PAGE gel use for the Western hybridization. Lane mw, SeeBlue⁷ Plus2 molecular weight markers; lane 1, *B. fragilis* 638R; lane 2, *B. fragilis* 638R + pFD1142 (1/20 dilution); lane 3 *B. fragilis* 638R + pFD1142; Lane 4, *E. coli* containing pSRKKm as a positive control. The 38 kDa band corresponding to LacI is indicated by the arrow to the right of the panel.

Fig. 3.

Functional map of the expression vector, pFD1146. The map shows the major features of the expression plasmid as grey block arrows for genes and promoters and rectangles for other sites. The replication gene, *repA*, is required for replication in *Bacteroides* and *ermF* is the *Bacteroides* erythromycin resistance gene. The RK2 *oriT* used for mobilization of the plasmid from *E. coli* to *Bacteroides* is shown and the *aad9* gene provides resistance to spectinomycin in *E. coli*.

Fig. 4. Growth characteristics of *B. fragilis* strains containing pFD1146 and related plasmids. Growth in BHIS was measured in 13×100 mm tubes at A_{550} at the intervals indicated. The parent strain 638R was grown without erythromycin and the plasmid containing strains were grown in the presence of 10 μ g/ml of erythromycin.

Fig. 5.

IPTG controlled expression of the xylosidase reporter gene in pFD1147. (A) Cultures of 638R containing pFD1147 were grown in BHIS media to mid-logarithmic phase (A_{550} 0.3) and then induced for 2 h with the concentration of IPTG indicated. Cells then were harvested by centrifugation and xylosidase assays were performed. (B) Cells were grown in BHIS media to mid-logarithmic phase (A_{550} 0.3) and then induced with 1.0 mM IPTG for the time indicated. Cells then were harvested by centrifugation and xylosidase assays were performed. All data were obtained from triplicate assays in two independent experiments.

Fig. 6.

Regulated expression of the *B. fragilis trxA* gene cloned into pFD1146. Western hybridization analyses of the cMyc-tagged *trxA* gene product with c-Myc antibody was used to measure induction following the addition of IPTG. Cells were grown in BHIS to mid-logarithmic phase (A_{550} 0.35), then induced as described below and cells electrophoresed on 15% SDS-PAGE gels. (A) Cells were induced with 1 mM IPTG for 0–24 h as indicated and then harvested by centrifugation. c=no induction prior to addition of IPTG. (B) Cells were induced for 3.5 h with the concentration of IPTG as indicated. The c-Myc tagged TrxA protein with a mass of 12,700 is indicated by the arrowhead on the right of the blot. A non-specific protein that reacts with the cMyc antibody but does not respond to IPTG addition is present above the cMyc-tagged TrxA protein.

Table 1

Strains and plasmids used in this study

strain or plasmid	relevant properties ^a	reference
Strains		
<i>B. fragilis</i> 638R	Wild type, clinical isolate, R ^r	(Privitera et al 1979)
<i>E. coli</i> EC100	laboratory strain, F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ ⁻ rpsL (Str ^r) nupG	Epicentre, Inc, Madison
Plasmids		
pFD288	8.9 kb, shuttle vector, Em ^r , (Sp ^r)	(Smith et al 1995)
pFD351	11.1 kb, pFD288 containing <i>cfxA</i> gene, Em ^r , Fx ^r , (Sp ^r)	(Parker and Smith 1993)
pFD1140	9.1 kb, pFD288 containing <i>lacO</i> _{1,3} modified <i>cfxA</i> promoter, Em ^r , (Sp ^r)	this study
pFD1142	10.5 kb, pFD288 containing 504 bp tetQ promoter fragment and <i>lacI</i> gene, Em ^r , (Sp ^r)	this study
pFD1146	9.4 kb, IPTG inducible <i>Bacteroides</i> expression vector, Em ^r , (Sp ^r)	this study
pFD1147	10.4 kb, pFD1146 containing the <i>XA</i> reporter gene cloned into BamHI site, Em ^r , (Sp ^r)	this study
pFD1153	9.8 kb, pFD1146 containing <i>trxA-cMyc</i> gene cloned into the BamHI and SacI sites, Em ^r , (Sp ^r)	this study
pSRKKm	5.8 kb, IPTG inducible <i>Proteobacteria</i> expression vector, (Km ^r)	(Khan et al 2008)

^aThe antibiotic resistance phenotypes expressed in *E. coli* are indicated by the designations enclosed by parentheses and *Bacteroides* phenotypes are shown without parentheses.

Table 2

Oligonucleotide primers used for construction of pFD1146

primer name	sequence ^a
cfxA promoter & lacO1 cloning	
Cfx-lacO1-R (Sall)	acgtg <i>tcgaca</i> aattgttatccgctcacaatt GCCGACAAAGGTACATAACTAAAG
Cfx-F (SphI)	acgtg <i>catgc</i> CATGATTGAAAGCTCATA
lacO3 cloning	
Cfx-lacO3-F (SphI)	acgtg <i>catgc</i> ggcagtgagcgcaacgcaatt TTACAAAGAAAATTCGACAAACTG
Cfx-R	CGAACTTAGTCATTTGAATG
lacI cloning	
Laci-F (Sall)	acgtg <i>tcgaca</i> aaataagaacaatt ATGAAACCAGTAACGTTATACGA
Laci-R (EcoRI)	acgtg <i>gaattc</i> AACTCACATTAATTGCGTTG
tetQ promoter +lacI cloning	
I-tet-F (EcoRI)	acgtg <i>gaattc</i> CCCAAAGGTCTAAAAGTAA
I-tet-R (SmaI)	acgtcccgggAACTCACATTAATTGCGTTG

^aThe primer sequences are shown 5' -3'. Uppercase letters designate sequences that match the template in question. Lowercase letters are additions to the 5' end. Sequence in italics shows the engineered restriction sites. Sequences in bold font show insertions of the lacO1 or lacO3 binding sites, or in the case of primer Laci-F the bold font is the *B. fragilis ahpC* ribosome binding site engineered upstream of the *lacI* ATG start site.