

学 位 論 文

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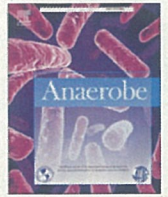
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Counterselection employing mutated *pheS* for markerless genetic deletion in *Bacteroides* species



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ABSTRACT

Markerless gene deletion is necessary for multiple gene disruptions due to the limited number of antibiotic resistant markers for some bacteria. However, even in transformable strains, obtaining the expected mutation without a marker requires laborious screening of a large number of colonies. Previous studies had success in various bacteria with a counter-selection system where a conditional lethal gene was incorporated into the vector. We examined the efficacy of the mutated *pheS* gene (*pheS*^{*}) as a counter-selective marker for gene deletion in *Bacteroides*. This mutation produces an amino acid substitution (A303G) in the alpha subunit of *Bacteroides* phenylalanyl tRNA synthetase, which in *E. coli* alters the specificity of the tRNA synthetase resulting in a conditional lethal mutation due to the incorporation of *p*-chloro-phenylalanine (*p*-Cl-Phe) into protein. *B. fragilis* YCH46 and *B. thetaiotaomicron* VPI-5482 transformed with a *pheS*^{*}-harboring shuttle vector were clearly growth-inhibited in the presence of >5 mM *p*-Cl-Phe in liquid defined minimal media (DMM) and on DMM agar plates. A targeting plasmid was constructed to delete the genetic region for capsular polysaccharide PS2 in *B. fragilis* or PS1 in *B. thetaiotaomicron*. After counterselection, *p*-Cl-Phe-resistant colonies were generated at a frequency of 8.1×10^{-3} for *B. fragilis* and 1.7×10^{-3} for *B. thetaiotaomicron*. Of the *p*-Cl-Phe-resistant colonies, 4.2% and 72% harbored the correct genetic deletion for *B. fragilis* and *B. thetaiotaomicron*, respectively. These results indicate that mutated *pheS* is a useful counter-selective gene to construct markerless genetic deletions in *Bacteroides*.

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1. Introduction

The human digestive tract harbors $\sim 10^{14}$ microbial cells, which is equivalent to human somatic cells number [1]. A consortium of these microbial cells constitute normal microflora that influence human physiology. The genus *Bacteroides* is among the predominant members of the normal human gut microflora. Metagenomic analyses and studies employing gnotobiotic mice are revealing that *Bacteroides* modulates a variety of human functions, such as gut epithelial cell proliferation and differentiation, gut immune

maturation, and energy metabolism [2,3]. One of the representative intestinal *Bacteroides* species, *B. thetaiotaomicron*, has been reported to induce various responses in the gut, including anti-inflammation by PPAR- γ -mediated suppression of NF- κ B translocation to the nucleus [4], induction of antimicrobial peptides [5] or IgA [6], and suppression of the virulence of enterohemorrhagic *Escherichia coli* [7]. From these findings, *B. thetaiotaomicron* is considered to be a stabilizer of gut microbiota and is used as a standard to analyze *Bacteroides*-host interaction [8].

We have previously reported successful in-frame genetic deletions in *B. fragilis* [9,10] and *mariner* transposon-based random mutagenesis in *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, and *B. vulgatus* [11]. However, markerless genetic deletion is still difficult in many *Bacteroides* strains. In our *B. fragilis* genetic deletion

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system, a suicide plasmid containing DNA fragments homologous to the target genetic site is integrated into the bacterial chromosome. The obtained integration mutant (merodiploid) is then cultured without selective antibiotics to remove vector sequence by a second homologous recombination. Finally, screening of several thousands of replica-plated colonies is needed to find a colony that has lost the vector sequence by second homologous recombination. This screening step is a time-consuming and laborious process.

Counterselection in bacterial gene deletion is an efficient way to reduce the time spent screening for the correct deletion mutant [12]. Genes that impart a lethal effect on the host strain under specific conditions (e.g. temperature or the addition of a substrate for the counter-selection gene product) are generally used for counterselection of the expected mutant. In *Bacteroides*, the thymidine kinase gene (*tdk*) and thymidylate synthetase (*thyA*) have been used as a counter-selection gene for these bacteria [20,21] since these bacteria are sensitive to 5-fluoro-2'-deoxyuridine and trimethoprim, respectively. However, efficiency of *tdk* and *thyA* counterselection are strain dependent, requiring first that each *Bacteroides* strain be tested for 5-fluoro-2'-deoxyuridine or trimethoprim sensitivity in *tdk* or *thyA* null mutants, respectively. At present, a counter-selection gene widely applicable for *Bacteroides* strains remains to be identified.

Recently, a mutant *pheS* gene (*pheS**), which encodes the α subunit of phenylalanyl-tRNA synthetase, has been reported to be useful for counterselection for markerless deletion in various species [13–19]. In *E. coli*, the mutant *PheS*^{A294G} displays broader substrate specificity than wild-type *PheS*, resulting in the incorporation of a halogenated analogue of phenylalanine, *p*-chlorophenylalanine (*p*-Cl-Phe), into tRNA. As a result, the cells harboring *pheS** die due to the toxicity of *p*-Cl-Phe [22,23].

In the current study, we developed a counter-selection system employing the mutant *pheS* gene to efficiently introduce markerless genetic deletions into the *Bacteroides* genome.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains used in this study were *Escherichia coli* DH5 α and two genome-sequenced *Bacteroides* strains, *B. fragilis* YCH46 and *B. thetaiotaomicron* VPI-5482. Plasmids used were *E. coli-Bacteroides* shuttle plasmids, pNLY1 [26], pVAL-1 [25], pLYL05 [26], and their derivatives containing *pheS** gene. The pBluescript KS II (+)-based pKK100 [10] was used as the suicide vector for allelic exchange in *Bacteroides* genomes. Detailed information for these bacterial strains and plasmids is summarized in Table S1.

2.2. Culture conditions

B. fragilis YCH46 and *B. thetaiotaomicron* VPI-5482 were cultured anaerobically in GAM broth (Nissui Pharmaceutical Co. Ltd., Tokyo) or on GAM agar plates at 37°C for 24 h. Defined minimal medium (DMM; containing only 0.075% methionine as an amino acid [24]) containing various concentrations of *p*-chloro-phenylalanine (*p*-Cl-Phe) was used as counter-selection medium for *Bacteroides* strains. Anaerobic cultivation was performed with the AnaeroPack system (Mitsubishi Gas Chemical Co. Ltd.) or an anaerobic chamber maintained with 80% N₂, 10% CO₂, and 10% H₂. Antibiotics were added to the media when necessary: ampicillin (Ap), 100 μ g/ml; cefoxitin (Cfx), 20 μ g/ml; chloramphenicol (Cm), 15 μ g/ml; erythromycin (Em), 10 μ g/ml; tetracycline (Tc), 15 μ g/ml.

2.3. Plasmid construction for *Bacteroides* transformation with *pheS**

The *B. fragilis* YCH46 *pheS* gene, including its promoter, was amplified by Prime Star polymerase (TAKARA) using primers *pheS*-(BF)-F and *pheS*-(BF)-R, which were designed to have *Pst*I sites at their ends. The amplicon was digested by *Pst*I and cloned into the *Pst*I site of *E. coli-Bacteroides* shuttle vector pLYL05, constructing pLYL05-*pheS*. Inverse PCR was performed with pLYL05-*pheS* as a template using primers *pheS*-inverse-F and *pheS*-inverse-R, which were designed to integrate the mutation into *pheS* (Fig. 1B). The amplicon was phosphorylated at both ends and self-ligated, constructing pLYL05-*pheS**. The *pheS** on pLYL05-*pheS** was amplified with primers, *pheS*-(BF)-F and *pheS*-(BF)-R, and then cloned into the *Sma*I site of pNLY1 and the *Nae*I site of pKK100, creating pNLY1-*pheS** and pKK100-*pheS** respectively. Primer sequences are listed in Table S2.

2.4. Growth monitoring of *Bacteroides* strains harboring mutated *pheS* in *p*-Cl-Phe-containing media

B. fragilis harboring pLYL05 or pLYL05-*pheS** was cultured overnight in DMM/Cfx (20 μ g/ml) broth. The growth of *B. fragilis* harboring pLYL05 or pLYL05-*pheS** in DMM/Cfx (20 μ g/ml) broth with or without *p*-Cl-Phe (5, 10, 15, or 20 mM) was monitored by measuring OD₅₉₀ of the culture every 3 h after the overnight culture was inoculated into the fresh media (2.0% v/v). Cytotoxicity of *p*-Cl-Phe to *B. thetaiotaomicron* harboring pNLY1 or pNLY1-*pheS** was also assessed as described for *B. fragilis*, except that 15 μ g/mlCm was used instead of 20 μ g/ml Cfx and 0.5–5 mM *p*-Cl-Phe was tested.

2.5. Assessing cytotoxicity of *p*-Cl-Phe to *Bacteroides*

B. fragilis YCH46 harboring pLYL05 or pLYL05-*pheS** and *B. thetaiotaomicron* VPI-5482 harboring pNLY1 or pNLY1-*pheS** were anaerobically cultured overnight at 37°C in GAM broth containing 20 μ g/ml Cfx and 15 μ g/mlCm, respectively. These cultures were diluted 10–10⁶-fold with phosphate-buffered saline (PBS, pH7.4), and the serial dilutions (5 μ l) were spotted onto GAM or DMM agar with or without *p*-Cl-Phe (5, 10, 15, 20, or 25 mM). The plates were anaerobically incubated for 48 h at 37°C before comparing the growth. To examine the killing effect of *p*-Cl-Phe, the cell suspensions of above mentioned *Bacteroides* strains in DMM containing selective antibiotics were anaerobically incubated with or without 20 mM *p*-Cl-Phe for 6 h. The viable cell numbers were determined by plating the appropriate dilutions of the cell suspensions onto GAM agar plates containing selective antibiotics. The colony on GAM plates was counted after 48-h anaerobic cultivation at 37°C.

2.6. Construction of *pheS* counter-selection vector for *Bacteroides*

The *Bacteroides pheS* counter-selection vector was constructed as follows. The amplified *pheS** was cloned into the *Nde*I/*Xba*I site of pVAL-Exp [10] to set the gene under control of the strong promoter *IS1224/cepA* [27], constructing pVAL-Exp*pheS**. The fragment containing *IS1224/cepA* promoter and *pheS** was amplified with primers pVAL-Nh/N-F and pVAL-S/N-R using pVAL-Exp*pheS** as a template. The amplicon was ligated to *Nae*I-digested pKK100, generating pKK100-Exp*pheS**.

Targeting vectors pKK100-Exp*pheS**-BFPS2 and pKK100-Exp*pheS**-BTPS1 were constructed as follows. PCR was used to amplify the 2-kb regions upstream and downstream of the capsular polysaccharide biosynthesis locus PS2 or PS1 in *B. fragilis* YCH46 and *B. thetaiotaomicron* VPI-5482, respectively. The upstream and

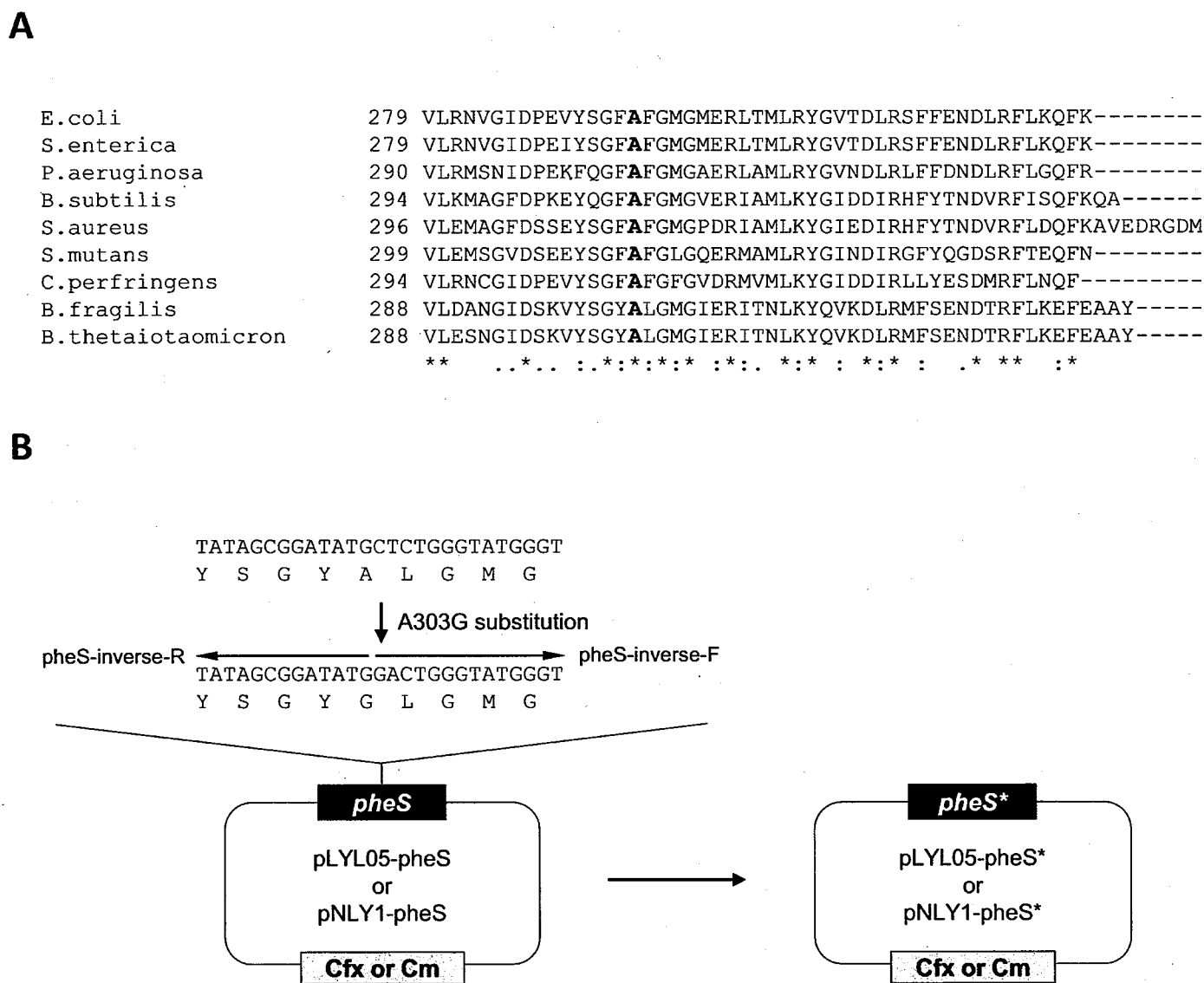


Fig. 1. Amino acid sequence alignment of PheS and generation of mutated *pheS*. The PheS protein is the α -subunit of phenylalanyl tRNA synthase and is conserved in many bacterial species. (A) The C-terminal amino acids of PheS from nine bacterial species were aligned with ClustalW. The alanine at position 294 in *E. coli*, which when substituted with glycine is known to be lethal in the presence of *p*-Cl-Phe, is conserved (shown in bold). (B) Schematic diagram of PheS counter selection vector construction. The *pheS* derived from *B. fragilis* YCH46 was cloned into *E. coli*-*Bacteroides* shuttle vectors pLYL05 or pNLY1 creating pLYL05-*pheS* or pNLY1-*pheS*, respectively. The wildtype *pheS* was changed to *pheS** using inverse PCR to introduce a codon exchange of GCT to GCA that caused the amino acid substitution A303G in PheS.

downstream regions were fused by PCR via the overlapping sequence incorporated into the primers. Finally, the two resulting products were each cloned into the *Xba*I site of pKK100-ExpheS*.

2.7. Integration of the targeting plasmid into the chromosome

Electrotransformation of *Bacteroides* strains with pKK100-ExpheS*-BFPS2 or pKK100-ExpheS*-BTPS1 was performed as described previously [9]. Competent cells of the *Bacteroides* strains were prepared from two different anaerobic culture stages in GAM broth at 37°C: early logarithmic phase ($OD_{660} = 0.2-0.4$) for *B. fragilis* YCH46 and stationary phase (48-h incubation) for *B. thetaiotaomicron* VPI-5482. The *Bacteroides* cells from the appropriate culture phase were collected by centrifugation, suspended in ice-cold 10% glycerol, and used as competent cells.

A mixture of 0.1 ml competent cells and 10 μ l of targeting

plasmids (pKK100-ExpheS*-BFPS2 or pKK100-ExpheS*-BTPS1) were transferred to 2 mm width electroporation cuvettes and electrically pulsed (setting: 12.5 kV/cm, 200 Ω , 25 μ F) with the Gene-Pulser II apparatus (Bio-Rad). After the pulse, bacterial cells were immediately transferred to 0.9 ml of pre-warmed GAM broth and anaerobically incubated at 37°C for 12 h. To select clones that had undergone homologous recombination to integrate the targeting plasmid, 0.1 ml of the culture was spread onto a GAM agar plate containing 10 μ g/ml Em. The clones obtained were designated BF::pKK100-ExpheS*-BFPS2 and BT::pKK100-ExpheS*-BTPS1, respectively.

2.8. Counterselection using *p*-Cl-Phe

To obtain the PS loci deletion mutant, BF::pKK100-ExpheS*-BFPS2 and BT::pKK100-ExpheS*-BTPS1 were anaerobically

cultured for 24 h in GAM broth without Em. The bacterial cells were collected by centrifugation, washed with PBS, re-suspended in PBS, and then 0.1 ml of the appropriate dilution was plated onto DMM agar supplemented with *p*-Cl-Phe (15 mM for *B. fragilis* YCH46; 10 mM for *B. thetaiotaomicron* VPI-5482). The colonies collected from the DMM/*p*-Cl-Phe plates after 72-h cultivation at 37°C were streaked on GAM and GAM/Em plate to check the sensitivity to Em. DNAs were purified from Em-sensitive colonies using the Easy-DNA kit (Invitrogen). After the DNA concentration was determined, PCR was performed using primers encompassing the deletion site and 100 ng of the purified DNA as template. The amplicon sizes were compared with that from the parental strain by agarose gel electrophoresis to confirm that the expected deletion occurred.

3. Results

3.1. Cytotoxicity of *p*-Cl-Phe to *Bacteroides* expressing mutated *pheS*

C-terminal amino acid sequences of PheS of *B. fragilis* YCH46 and *B. thetaiotaomicron* VPI-5482 were aligned with those of other species (Fig. 1A). The amino acid substitution of alanine 294 with glycine in *E. coli* PheS is known to reduce the substrate specificity of phenylalanyl-tRNA synthetase. This allows the incorporation of chlorinated phenylalanine analogue (*p*-Cl-Phe) into proteins, resulting in cell death. Based on the alignment, this alanine residue was also conserved at position 303 in *Bacteroides* phenylalanyl-tRNA synthetase.

Cytotoxicity of *p*-Cl-Phe to *Bacteroides* expressing an A303G mutant PheS (PheS*, codon exchange from GCT to GGA) was tested. The wild-type *pheS* was first cloned into shuttle vector pLYL05 or pNLY1 to create pLYL05-*pheS* and pNLY1-*pheS*. The codon exchange to generate *pheS** was achieved with inverse PCR (Fig. 1B). pLYL05-*pheS** or pNLY1-*pheS** were introduced into *B. fragilis* YCH46 or *B. thetaiotaomicron* VPI-5482 by electroporation, respectively.

Bacteroides strains with the wild-type *pheS* gene (vector control) showed no significant difference in growth in liquid DMM with or without *p*-Cl-Phe (Fig. 2A and C). The growth of *B. fragilis* harboring *pheS** was clearly inhibited in a dose-dependent manner by *p*-Cl-Phe (Fig. 2B). Growth slowed in the presence of 5 mM *p*-Cl-Phe when compared with vector control, and was completely inhibited at concentrations of 15 mM and above. *B. thetaiotaomicron* harboring *pheS** was also inhibited dose-dependently by *p*-Cl-Phe, and the apparent growth inhibition was observed at 5 mM (Fig. 2D). This growth inhibition was not derived from bacterial killing because only small reduction was observed in the viable *Bacteroides* strains after 6-h exposure to 20 mM *p*-Cl-Phe (Fig. S1).

To find the optimal *p*-Cl-Phe concentration for counterselection on agar plates, 10-fold serial dilutions of overnight culture of *Bacteroides* strains expressing *pheS** were spotted onto GAM or DMM agar plates supplemented with 5–25 mM *p*-Cl-Phe. No difference was observed between the growth of *B. fragilis* expressing *pheS** and vector control on GAM agar plates containing *p*-Cl-Phe, even at high concentrations (Fig. 3A). For *B. thetaiotaomicron* VPI-5482, growth of strains expressing *pheS** or vector control were the same on GAM agar plates containing up to 15 mM of *p*-Cl-Phe; at 20 mM or more, growth of the *pheS** strain was inhibited (Fig. 4A). On the other hand, both *Bacteroides* strains expressing *pheS** were clearly inhibited on DMM agar plates with as low as 5 mM *p*-Cl-Phe when compared with the vector control (Figs. 3B and 4B). The inhibitory effect of *p*-Cl-Phe on the growth of *Bacteroides* strains harbouring *pheS** was decreased by phenylalanine supplementation (Fig. S2). These results indicate that nutritionally restricted media should be used for *pheS** counterselection in *Bacteroides*.

3.2. Plasmid design for *pheS* counterselection

A schematic map of the counter-selection vector, pKK100-ExpheS*, constructed in this study is shown in Fig. 5A. Suicide plasmid pKK100 was used for the backbone since it already contained a *Bacteroides* antibiotic-resistance marker, *ermF*. The *pheS** gene was connected to the strong IS1442/*cepA* promoter from *B. fragilis* RBF103 [27,28] by traditional PCR cloning methods, and then the DNA fragment was cloned into the *NheI/XbaI* site of pKK100. To target specific *Bacteroides* genes for deletion, PCR fragments that are homologous to the upstream and downstream DNA sequences surrounding the gene of interest are cloned into the multiple cloning site (MCS).

3.3. Deletion of capsular polysaccharide biosynthesis gene loci

Capsular polysaccharides are major virulence factors for *Bacteroides* species [29]. Among a number of capsular polysaccharide biosynthesis (PS) loci, the PS2 of *B. fragilis* YCH46 and PS1 of *B. thetaiotaomicron* VPI-5482 were targeted for deletion using *pheS** counterselection because their roles in virulence or gut colonization are still uncharacterized so far. Nearly 2-kb each of the upstream and downstream regions of the PS loci were used as the targeting sequence for the suicide vectors (pKK100-ExpheS*-BFPS2 and pKK100-ExpheS*-BTPS1). As diagramed in Fig. 5B, the targeting vectors were integrated into the genome of the respective *Bacteroides* strain, generating the merodiploid strains BF::pKK100-ExpheS*-BFPS2 and BT::pKK100-ExpheS*-BTPS1. The merodiploids were grown overnight without antibiotics before counterselection on DMM agar plates containing 15 mM (for *B. fragilis*) or 10 mM (for *B. thetaiotaomicron*) *p*-Cl-Phe. *p*-Cl-Phe-resistant colonies were generated from BF::pKK100-ExpheS*-BFPS2 and BT::pKK100-ExpheS*-BTPS1 at frequencies of 8.91×10^{-3} and 1.70×10^{-3} , respectively. All of the colonies grown on DMM/*p*-Cl-Phe agar were also Em-sensitive (no false positives), indicating that the counterselection was successful.

3.4. Confirmation of genetic deletion in PS loci

It was possible that some of the Em-sensitive clones obtained after counterselection reverted to the wild genotype. To verify that the PS loci was removed, PCR was performed using primers that surrounded the 21-kb fragment in PS2 of *B. fragilis* YCH46 or the 27-kb fragment in PS1 of *B. thetaiotaomicron* VPI-5482 (Fig. 6A and B). Of the 96 Em-sensitive clones obtained by *pheS** counterselection, 4.17% of *B. fragilis* clones and 72.2% of *B. thetaiotaomicron* clones showed the expected deletion while all of the remaining colonies were revertants to wild genotypes.

4. Discussion

Gene disruption is an initial step to identify the physiological function of a gene of interest. Bacterial isolates from clinical specimens or environmental samples are often difficult to genetically manipulate due to resistance to foreign DNAs. Even in the case of transformable bacteria, gene disruption is often limited to integration of targeting vector. However, analysis of these integration mutants must take into account the effect of the antimicrobial resistant marker on gene expression and any possible polar effects on the downstream genes.

Bacteroides is among the bacteria that are difficult to genetically manipulate. So far, we have reported efficient electrotransformation [10] and in-frame markerless genetic deletion [9,10] for *B. fragilis*. However, one-step gene replacement (double crossing over) as in *E. coli* laboratory strains is still difficult in *B. fragilis*.

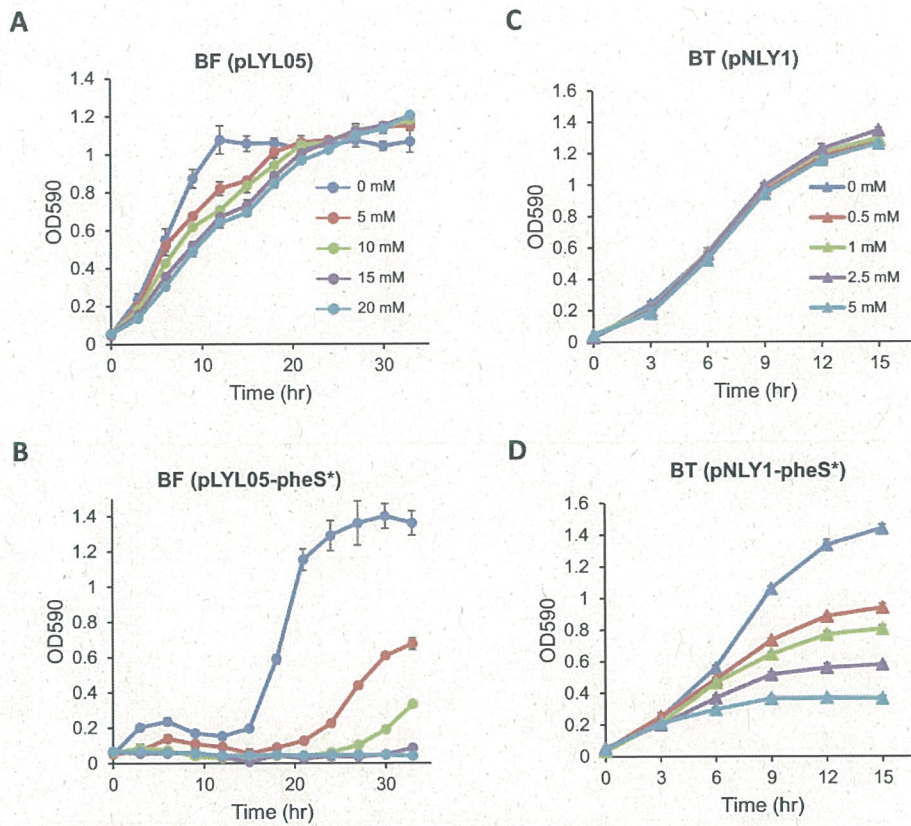


Fig. 2. Growth in liquid culture of *Bacteroides* strains transformed with *pheS** is inhibited by *p*-Cl-Phe. Growth curves of *B. fragilis* YCH46 (A and B) and *B. thetaiotaomicron* VPI-5482 (C and D) harboring a control vector (A and C) or vector containing *pheS** (B and D). The *Bacteroides* strains were grown in DMM/Cfx broth or DMM/Cm broth with or without *p*-Cl-Phe (0–20 mM). *p*-Cl-Phe concentrations are indicated by colored symbols.

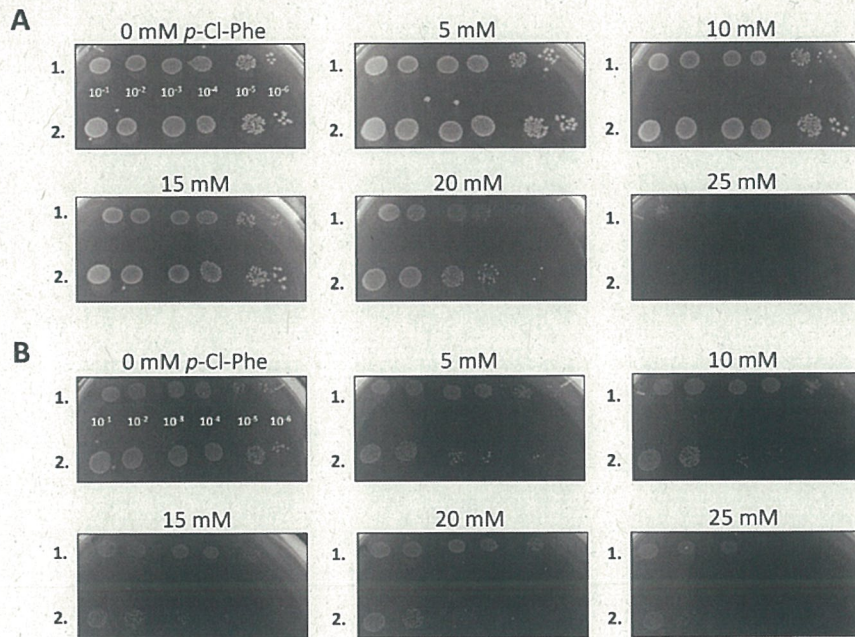


Fig. 3. Effect of *p*-Cl-Phe on growth in agar plates of *B. fragilis* transformed with *pheS**. Serial dilutions of overnight cultures of *B. fragilis* YCH46 harboring control vector (row 1) or pLYL05-*pheS** (row 2) were prepared, and 5 μ l of each dilution was spotted onto GAM/Cfx (panel A) or DMM/Cfx (panel B) agar plates supplemented with indicated concentrations *p*-Cl-Phe.

Therefore, in-frame markerless deletion in *B. fragilis* is composed of two steps: 1) integration of targeting vector into the chromosome,

and 2) screening of the clones without antibiotic selection to identify those with the expected in-frame deletion. The second step

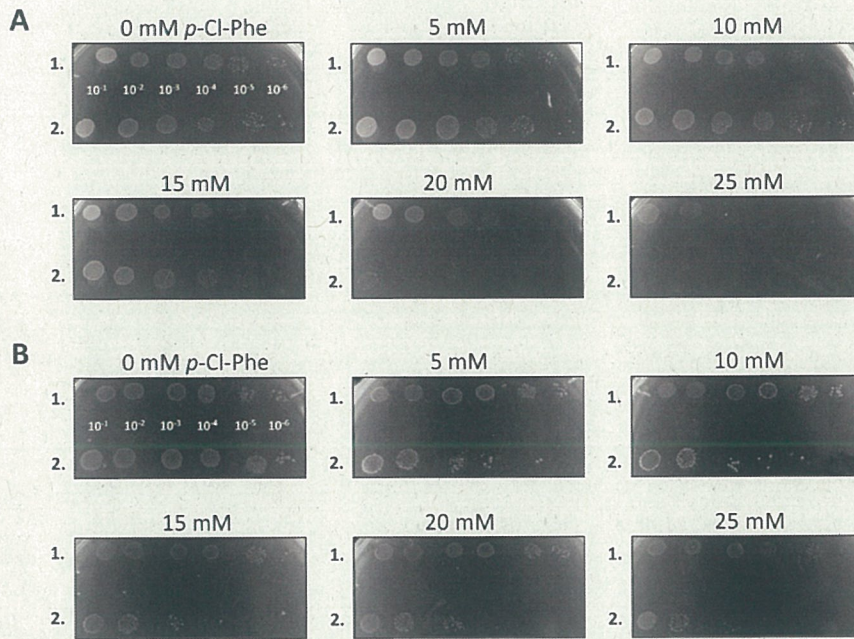


Fig. 4. Effect of *p*-Cl-Phe on growth in agar plates of *B. thetaiotaomicron* transformed with *pheS**. Serial dilutions of overnight cultures of *B. thetaiotaomicron* VPI-5482 harboring control vector (row 1) or pNLY1-*pheS** (row 2) were prepared, and 5 μ l of each dilution was spotted onto GAM/Cm (panel A) or DMM/Cm (panel B) agar plates supplemented with indicated concentrations *p*-Cl-Phe.

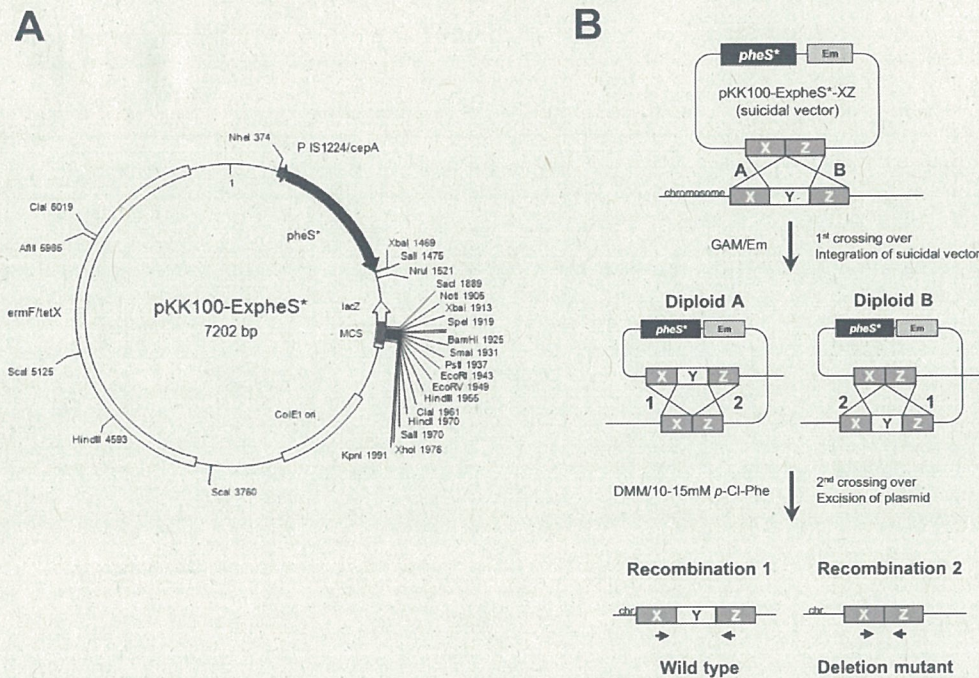


Fig. 5. *pheS** counter-selection strategy for markerless genetic deletion in *Bacteroides*. (A) Plasmid map of the counter-selection vector pKK100-ExpheS* used for gene deletion in *Bacteroides*. The strong hybrid *Bacteroides* promoter IS1224/*cepA* was used to express *pheS**. *pheS** was placed downstream of the multiple cloning site (MCS), where the chromosome targeting sequence is inserted. The *ermF* gene confers resistance to erythromycin in *Bacteroides*, which is used for the first round of selection. The vector also contains elements for propagation in *E. coli*: ColE1 origin of replication and the tetracycline resistance gene, *tetX*. (B) Schematic diagram for deletion of PS2 in *B. fragilis* and PS1 for *B. thetaiotaomicron* using *p*-Cl-Phe counterselection. The counter-selection vector described in (A) harboring 2-kb fragments (X and Z) homologous to the areas adjacent to PS2 or PS1 (Y) was integrated into the *Bacteroides* chromosome by homologous recombination (HR) due to pressure from Em selection. Two merodiploids can result from the HR (diploid A or B). Em-resistant colonies were subjected to counterselection by cultivation on DMM agar containing *p*-Cl-Phe. Only colonies that undergo a second HR event to remove the vector will survive. *p*-Cl-Phe-resistant colonies will either have the PS loci removed or have reverted to the wildtype.

is laborious since an extensive number of colonies (generally 10,000–20,000 in our laboratory) must be screened to obtain a candidate clone, and we often find that all clones from an

experiment are revertants to wildtype.

Counter-selection systems incorporating a suicide vector, which produces a toxic effect to host cells under specific conditions, have

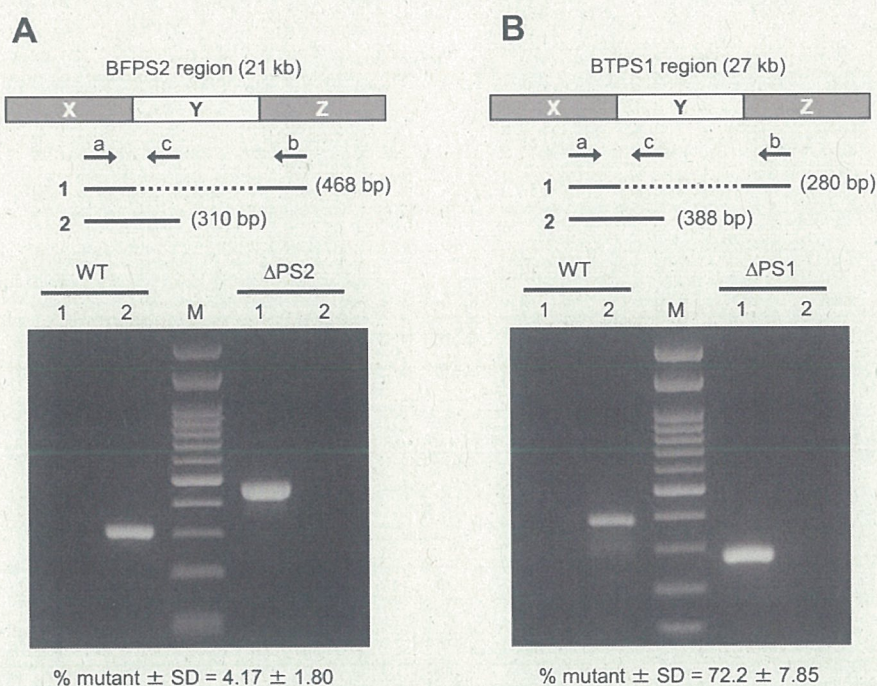


Fig. 6. Conformation of deletion of the PS2 loci of *B. fragilis* (A) and PS1 loci of *B. thetaiotaomicron* (B). PCR followed by agarose gel electrophoresis was performed to verify deletion of the PS loci. Two primers were located outside of the PS loci (a and b), and one was located within the PS loci (c). If the PS loci were present (WT), then PCR with a and b primers would not amplify due to the length of the PS loci and PCR with a and c would produce small amplicons (310-bp for *B. fragilis* PS2 and 388-bp for *B. thetaiotaomicron* PS1). If the deletion occurred (Δ PS), PCR using primers a and b would produce a fragment of 468-bp or 280-bp from *B. fragilis* clones and *B. thetaiotaomicron* clones, respectively. PCR with primers a and c would show no amplicon if the PS loci was deleted. M indicates a size marker of 100-bp DNA ladder.

been reported as an efficient way to save the labor associated with screening. Among the counter-selection genes reported, *sacB*, which encodes levan sucrose, is widely used [12]. Levan sucrose produces levan-type water-insoluble fructan from sucrose, and the accumulation of fructan is toxic to host cells. The *sacB* gene was first identified in *Bacillus subtilis*, and it is not toxic for Gram-positive bacteria. On the other hand, introduction of *sacB* into *sacB*⁻ Gram-negative bacteria is toxic in the presence of high concentrations of sucrose due to accumulation of levan in the periplasmic space [12,30]. *Bacteroides* is a Gram-negative anaerobe with no *sacB* gene. However, introduction of a plasmid harboring *sacB* was not toxic to *B. fragilis* (data not shown). This is probably because *Bacteroides* produce many saccharolytic enzymes to utilize otherwise undigestible dietary fiber in the lower gut, including β -fructofuranosidases which degrades levan [31]. As for other counter-selection genes, *galk*, encoding galactokinase, *tdk*, encoding thymidine kinase, and *thyA*, encoding thymidylate synthetase, have been successfully applied to markerless genetic deletion in *Clostridium perfringens* [32], *B. thetaiotaomicron* [21], and *B. fragilis* [20], respectively. The disadvantage of these counter-selection systems is that they require a complementary strain where the corresponding chromosomal gene is removed.

Counter-selection systems employing *pheS*^{*} have recently been reported to be useful for genetic deletion in several pathogenic bacteria [13–19]. We demonstrated that *B. fragilis* and *B. thetaiotaomicron* transformed with plasmids harboring *pheS*^{*} (producing amino acid substitution of A303G in PheS) became sensitive to *p*-Cl-Phe, indicating that *pheS*^{*} counterselection is possible in *Bacteroides*. Furthermore, we employed *pheS*^{*} counterselection and obtained Em-sensitive clones at frequencies of $1.70\text{--}8.91 \times 10^{-3}$ without replica screening. In previous studies that did not include a second selection step, we were able to get the targeting vector to integrate into the expected site in

B. thetaiotaomicron VPI-5482 chromosome, but were not able to obtain the in-frame deletion mutant in this strain (data not shown). However, in this study, a PS1 deletion mutant was obtained in *B. thetaiotaomicron* VPI-5482 with *pheS*^{*} counterselection. These results indicate that *pheS*^{*} counterselection is a useful tool for in-frame markerless genetic deletion in *Bacteroides*.

In this study, we used defined minimal medium (DMM) for the selection of cells with *p*-Cl-Phe. In general, *Bacteroides* growth on DMM agar was slow and the colony size was small. Rich media (GAM, BHI, or PY agar plate) were tested; however, the toxic effect by *p*-Cl-Phe was weak, which resulted in all Em-resistant merodiploid colonies when grown on rich agar plates supplemented with 25 mM *p*-Cl-Phe (data not shown). High phenylalanine content in rich media probably canceled the toxic effect of *p*-Cl-Phe [13,33]. In fact, phenylalanine supplementation reduced the cytotoxicity of *p*-Cl-Phe to the *Bacteroides* expressing mutant *pheS* (Fig. S2). Developing a phenylalanine-restricted rich media would save labor for media preparation and improve bacterial growth in these experiments. Alternatively, an increase in the expression level of *pheS*^{*} could enable selection with *p*-Cl-Phe on rich media [16]. In this study, *pheS*^{*} was cloned under control of the strong IS1442/*cepA* promoter [27] but the expression level seemed to be insufficient to induce the toxicity of *p*-Cl-Phe on rich media. Finding highly inducible plasmids for *Bacteroides* may enable the *pheS* counterselection on rich media. It has been reported in *E. coli* that double amino acid substitutions of A294G and T251A or T251S increased the sensitivity to *p*-Cl-Phe [33]. In *PheS* of *B. fragilis* and *B. thetaiotaomicron*, the threonine residue is also conserved (at 249 in BF and BT). This doubly mutated *pheS*^{**} might also increase the efficiency of screening for fully resolved clones on rich media.

Homologous recombination between vector-cloned *pheS*^{*} and chromosomal *pheS* has been reported as a disadvantage of *pheS* counterselection [15,16,18]. However, in the current study, we did

not encounter such a case. It is possible our system avoided undesired homologous recombination because the targeting sequences used in this study (two 2-kb fragments) were much larger than the *pheS* gene (1.02 kb). For situations where *pheS* homologous recombination is an issue, introduction of a synonymous mutation into vector-cloned *pheS** is a way to overcome this problem [15,16,18].

5. Conclusions

Bacteroides provide beneficial physiological effects to human colonic epithelial cells, the immune system, and systemic metabolism. The genetic basis of these effects remains unclear, though some were strain-dependent. Recent advances in genome sequencing technology enable whole genome comparisons among a large number of isolates. These comparative genomics help identify candidate genes responsible for the function of interest, and multiple in-frame markerless genetic deletions is a necessary tool to understand these gene functions. The *pheS* counter-selection system reported here will accelerate the genetics in *Bacteroides* as well as further the discovery of health-promoting *Bacteroides* genes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anaerobe.2016.09.004>.

Conflict of interest

Authors declare no conflict of interests.

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