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PhoB Regulates the Survival of *Bacteroides fragilis* in Peritoneal Abscesses

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Abstract

In response to phosphate limitation, bacteria employ the Pho regulon, a specific regulatory network for phosphate acquisition. The two-component signal transduction system of PhoRB plays a crucial role in the induction of Pho regulon genes, leading to the adaptation to phosphate starvation. Herein, we identified the PhoRB system in *Bacteroides fragilis*, a commensal gut bacterium, and evaluated its role in gut colonization and survival in peritoneal abscesses. BF1575 and BF1576 encoded PhoR (sensor histidine kinase) and PhoB (response regulator) in the sequenced *B. fragilis* strain YCH46, respectively. Transcriptome analysis revealed that deletion of *phoB* affected the expression of 585 genes (more than 4-fold change) in *B. fragilis*, which included genes for stress response (chaperons and heat shock proteins), virulence (capsular polysaccharide biosynthesis) and phosphate metabolism. Deletion of *phoB* reduced the ability of the bacterium to persist in peritoneal abscesses induced by an intra-abdominal challenge of *B. fragilis*. Furthermore, PhoB was necessary for survival of this anaerobe in peritoneal abscesses but not for *in vitro* growth in rich media or in intestinal colonization. These results indicate that PhoB plays an important role in the survival of *B. fragilis* under stressful extraintestinal conditions.

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Introduction

Environmental sensing and adaptive response are essential activities for all organisms. Two-component signal transduction systems, composed of sensor protein and cognate response regulator, are commonly used for environmental sensing in bacteria [1]. Environmental stimuli trigger the histidine-aspartate phosphorelay of a phosphate group from an autophosphorylated sensor to a response regulator, resulting in the expression of the particular set of genes necessary for adaptive responses. The PhoRB system of *Escherichia coli* is a well characterized two-component system that senses inorganic phosphate (Pi) concentration in the external milieu [2]. PhoR is a sensor histidine kinase that phosphorylates PhoB, the response regulator, in conditions of low environmental Pi (less than 4 μM in the case of *E. coli*). Phospho-PhoB in turn regulates transcription of the Pho regulon, a large set of genes that are generally involved in phosphate homeostasis. PhoB has been associated with bacterial survival, stress response and virulence [3]. Induction of the Pho regulon has been reported during infections of *Yersinia pestis*, *Erwinia chrysanthemi*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* in diverse models [4–8].

The mammalian gut harbors a wide variety of microbes that must sense and respond to changes in nutritional availability, host immunity and microbial composition [9,10]. Post-operative injury leads to the release of host physiological stress products into the intestinal tract, where they directly activate the molecular circuitry of colonizing nosocomial pathogens, shifting their phenotypes to those of proinflammatory and lethal strains [11,12]. It has also been shown that intestinal Pi levels decrease after surgical operations such as 30% hepatectomy [13]. Operative injury-induced intestinal Pi depletion shifted the phenotype of *Pseudomonas aeruginosa* to express enhanced virulence in mice, resulting in exotoxin A permeability and death [11]. Pi repletion protected mice from gut-derived sepsis by *P. aeruginosa*. *Bacteroides*, a gram-negative obligate anaerobe, is one of the most predominant genera of gut microbiota. *B. fragilis* is among the more virulent species in the genus *Bacteroides*, and is frequently isolated from intra-abdominal infections such as peritonitis and peritoneal abscess [14–16]. *Bacteroides* infections often occur after intra-abdominal surgery. It is possible that alterations of intestinal parameters like Pi concentration and other environmental cues at extra-intestinal sites induce a shift from gut symbiosis to pathogenicity.

In this study, we identified the two-component signal transduction system corresponding to PhoRB in the sequenced *B. fragilis* strain YCH46 and evaluated the role of the *B. fragilis* PhoB on virulence in the intra-abdominal cavity and survival in peritoneal abscesses. The data indicated that PhoB shapes part of the molecular circuitry used by *B. fragilis* for survival in stressful environments.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *B. fragilis* strains were grown anaerobically at 37°C in Gifu Anaerobic Medium (GAM; Nissui Pharmaceutical Co., Tokyo, Japan) or on GAM agar plates using the AnaeroPack System (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) or an anaerobic chamber conditioned with mixed gases (N_2, 80%; CO_2, 10%; and H_2, 10%). To simulate the Pi-limiting conditions, defined minimal media (DMM) [17] was supplemented with varying concentrations of KH_2PO_4 (final concentration: 6.6, 0.066, and 0.0066 mM). *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar plates. If necessary, antibiotics were added to the media at the following concentrations: ampicillin, 50 μg/ml; cefoxitin, 50 μg/ml; erythromycin (Em), 10 μg/ml; and tetracycline, 10 μg/ml.

B. fragilis Gene Deletion

Deletion mutants for the *phoR* homolog (BF1575), *phoB* homolog (BF1576), or BF2185 were constructed in the *B. fragilis* strain YCH46 by removing the internal segment of each target gene. Briefly, DNA fragments upstream and downstream of the region being deleted were separately PCR-amplified and fused by a second PCR amplification via an overlapping regions incorporated into the primer sequences. The resultant PCR products were ligated into pKK100 [18,19]. The targeting plasmids were electroporated into *B. fragilis* strain YCH46 as described previously [18,19]. The diploids, in which targeting plasmid integrated into the chromosome via a single genetic crossover, were selected on GAM agar plates containing Em. The diploids were grown in GAM broth, spread on nonselective GAM agar plates, and replica plated to GAM agar plates containing Em to screen for mutants that resolved the diploid through the second homologous recombination. Em-sensitive colonies were selected, and the presence of the appropriate deletion was checked by PCR with primer pairs that flanked the deletion site (Table S1). The *phoB* gene was amplified using the *B. fragilis* YCH46 genome as the template and cloned into the modified *E. coli-Bacteroides* shuttle plasmid pLYL05-Exp (Table 1), generating pLYL1576. pLYL05-Exp or pLYL1576 were introduced into the *phoB* deletion mutant for complementation analyses. Synthetic oligonucleotide primers were purchased from Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan). The nucleotide sequences of all of the oligonucleotide primers used in this study are listed in Table S1. DNA sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (version 1.1; Applied Biosystems).

Growth Monitoring in Pi-limiting Conditions

B. fragilis strains were grown anaerobically in 10 ml of GAM broth at 37°C overnight (stationary phase). The cells were collected by centrifugation at 4°C, washed three times with 10 ml of DMM supplemented with 0.0066 mM KH_2PO_4, and resuspended in 10 ml of the same medium. The cell suspension (100 μl) was then inoculated into 10 ml of pre-warmed DMM (37°C) supplemented with varying concentrations of KH_2PO_4 (6.6, 0.066, or 0.0066 mM) and incubated anaerobically at 37°C. Growth was monitored over time by measuring the optical density at 600 nm. All manipulations were carried out in an anaerobic chamber. The doubling time of each strain was calculated from the optical densities at the two points of exponentially growing phase.

RNA Isolation and Quantitative PCR (qPCR)

B. fragilis cells grown to stationary phase in GAM were washed three times with DMM containing 0.0066 mM of KH_2PO_4 and diluted 100-fold with DMM plus 6.6 mM or 0.0066 mM of

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
Escherichia coli		
DH5α	F⁻ Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ⁻ *thi-1 gyrA96 relA1*	Laboratory strain
BL21	F⁻ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm*	Laboratory strain
Bacteroides fragilis		
YCH46	Clinical isolate, parental strain for all deletion mutants	37
TCSM1575 (ΔBF1575)	YCH46 mutant with deletion of BF1575	This study
TCSM1576 (ΔBF1576)	YCH46 mutant with deletion of BF1576	This study
TCSM2185 (ΔBF2185)	YCH46 mutant with deletion of BF2185	This study
Plasmids		
pKK100	Suicide vector for *Bacteroides*; Tc^R in *E. coli*, Em^R in *Bacteroides*	19
pLYL05	*E. coli-Bacteroides* shutle vector; Amp^R in *E. coli*, Cfx^R in *Bacteroides*	38
pLYL05-Exp	*Bacteroides* expression vector, IS*1224/cepA* hybrid promoter (39), restriction sites (*Nde* I, *Not* I, *Xba* I and *Sal* I), and the transcription terminator of BF1719 cloned into the *Kpn I/Sph* I site of pLYL05	This study
pLYL1576	BF1576 amplified with primers phoB-NdeI and phoB-XbaI from *B. fragilis* strain YCH46 cloned into the *Nde I/Xba* I site of pLYL05-Exp	This study
pGEX-6P-1	GST gene fusion vector, Amp^R	GE Healthcare
pGEX-phoB	BF1576 amplified with primers phoB-BamHI and phoB-R from *B. fragilis* strain YCH46 cloned into the *Bam*H I/*Sma* I site of pGEX-6P-1	This study

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KH_2PO_4. These dilutions were grown at 37°C under anaerobic conditions. Total RNA was extracted from mid-logarithmic phase cultures (OD_{660}; 0.4–0.6) using the hot-phenol method [20]. The RNA was further purified using an RNeasy CleanUp Kit (Qiagen) and treated with TURBO DNA-*free* (Ambion) to remove contaminating DNA. Total RNA (500 ng) was reverse transcribed using a SYBR ExScript RT-PCR Kit (Takara Shuzo Co., Ltd., Otsu, Japan) with random hexamers at 42°C for 15 min. Reverse transcription was terminated by heating the mixtures at 95°C for 2 min. The cDNA products were subsequently amplified using SYBR Premix Ex Taq (Takara) under the following conditions: preheating at 95°C for 10 s and 40 cycles of 95°C for 5 s and 60°C for 34 s in an ABI PRISM 7500 (Applied Biosystems). All samples were run in triplicate. Threshold cycle values were normalized with the levels of *rpoD* transcripts, and the changes were calculated by the $2^{-\Delta\Delta CT}$ method [21].

Microarray Analysis

We employed a *B. fragilis* YCH46 DNA microarray from NimbleGen Systems, which includes 4,527 target genes with at least 8 unique probes consisting of 60-mer synthetic oligonucleotides for each gene. The cDNA synthesis, hybridization, and scanning were performed by NimbleGen. Microarray data were analyzed by quantile normalization and robust multiarray averaging [22]. The normalized data were processed with ArrayStar software (DNASTAR). Samples were filtered to identify differential expression (over 4-fold change during Pi starvation compared to Pi excess). The Student's *t* test for the analysis of the mean log ratios of two samples and the subsequent Bonferroni adjustment for multiple testing were applied as rigorous criteria for significant changes in signal intensity. Changes with a *p*-value less than 0.05 ($p<0.05$) were considered statistically significant.

Expression and Purification of Recombinant PhoB

BF1576 was amplified by PCR and cloned into the pGEX-6P-1 expression vector (GE Healthcare) via fusion to glutathione-S-transferase (GST) gene, generating the plasmid pGEX-phoB (Table 1). *E. coli* strain BL21 harboring pGEX-phoB was grown in 100 ml of LB broth containing sorbitol (0.5 M) and ampicillin (50 μg/ml) at 37°C. IPTG was added to the culture at a final concentration of 0.1 mM when OD_{660} reached 0.3. The cells were further incubated at 25°C overnight. The cells were collected by centrifugation and resuspended with Cell Lytic B (Sigma) containing a protein inhibitor cocktail, lysozyme (200 μg/ml) and DTT (1 mM). Following a 15-min incubation at room temperature, the lysate was sonicated on ice and centrifuged at 13,000×*g* for 10 min at 4°C. The supernatant was incubated with glutathione sepharose 4B slurry (GE Healthcare) overnight at 4°C. The resin was washed five times with PBS-T wash buffer (1× phosphate-buffered saline, 0.5% Triton X-100). Proteins bound to the resin were eluted with 200 μl of glutathione elution buffer (50 mM Tris-HCl [pH8.0], 10 mM glutathione). Glutathione elution buffer was replaced by EMSA buffer (50 mM Tris-HCl [pH7.5], 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol) using Zeba Desalt Spin Columns (Thermo Scientific). The purity of recombinant PhoB was checked by SDS-PAGE.

Electrophoretic Mobility Shift Assay

Recombinant PhoB and 10 ng of bait DNA were mixed in binding buffer (50 mM Tris-HCl [pH7.5], 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA) and incubated for 30 min at 25°C. The predicted promoter regions of *pstC* (BF2756) and capsular

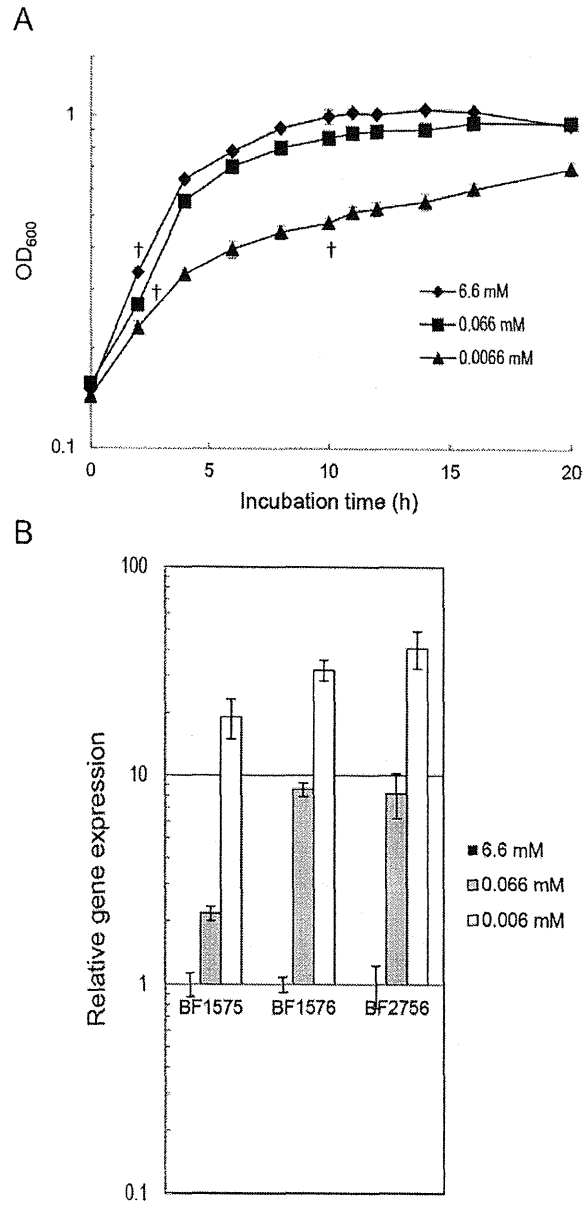

Figure 1. Response of *B. fragilis* to Pi limitation. (A) Growth curve in DMM supplemented with varying concentrations of KH_2PO_4. *B. fragilis* strain YCH46 was grown anaerobically in DMM supplemented with 6.6 mM, 0.066 mM, or 0.0066 mM of KH_2PO_4 at 37°C for 20 h. Optical densities of the cultures at 600 nm were measured over time. Data presented are the mean ± standard deviations of three independent cultures. (B) qPCR analysis of BF1575, BF1576, and BF2756. Total RNA was collected at mid-logarithmic phase (indicated by † in panel A). Expression levels of BF1575 (*phoR* homolog), BF1576 (*phoB* homolog), and BF2756 (*pstC* homolog) in wild type *B. fragilis* strain YCH46 were measured and normalized with transcriptional levels of *rpoD*. The transcriptional level of each gene under Pi limitation (0.066 mM or 0.0066 mM) is shown relative to that in DMM supplemented with 6.6 mM KH_2PO_4 (Pi-rich media).
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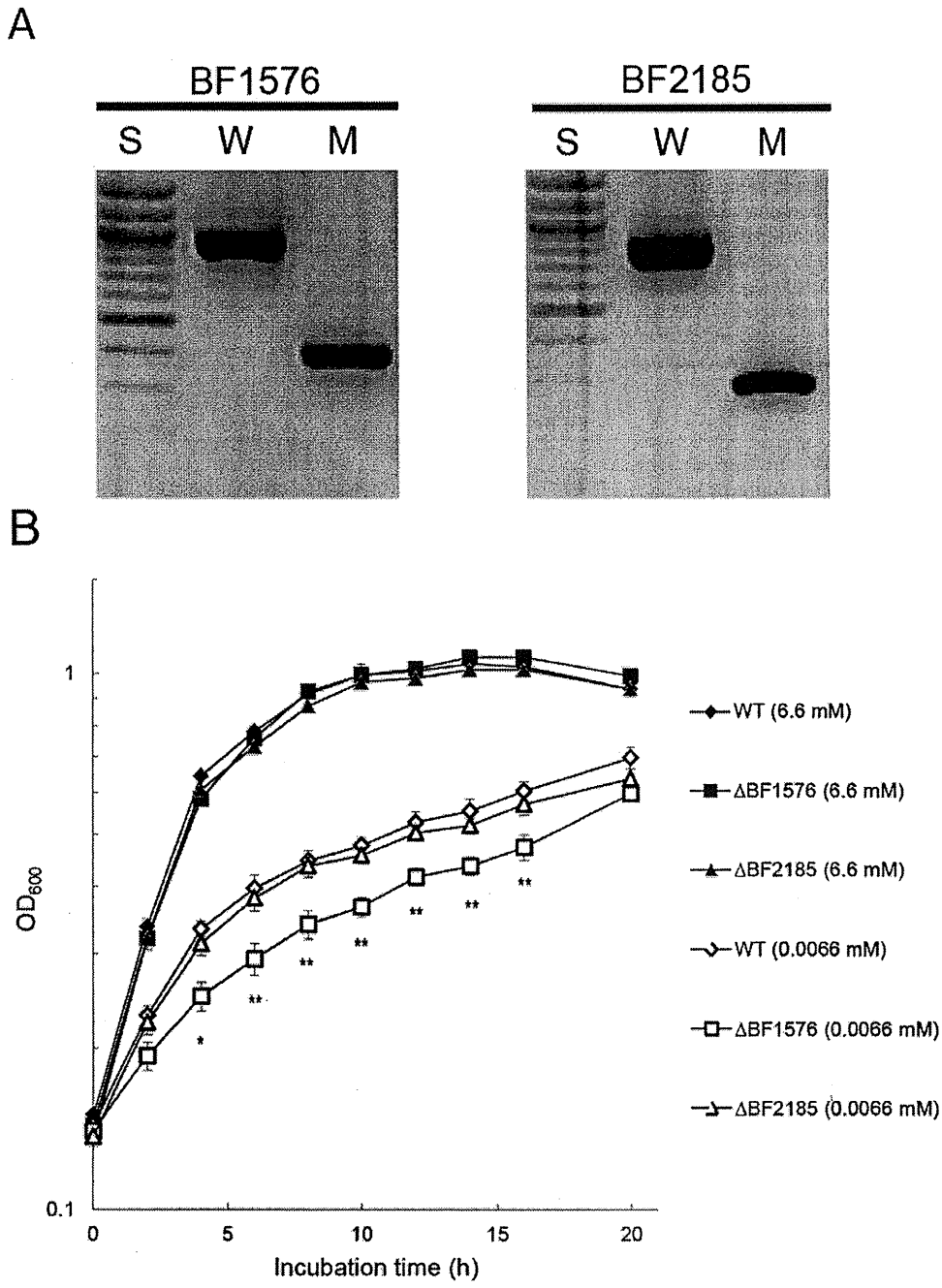

Figure 2. Effect of BF1576 deletion on the growth in Pi-limiting media. (A) Construction of ΔBF1576 and ΔBF2185 strains. Internal deletion of each gene was confirmed by PCR using a primer pair encompassing each deletion site. W, wild type *B. fragilis*; M, mutant form of indicated gene; S, 100-bp ladder size markers. (B) Growth of wild type (WT), ΔBF1576 and ΔBF2185 strains under Pi-rich and Pi-limiting conditions. WT (diamonds), ΔBF1576 (squares), and ΔBF2185 (triangles) strains of *B. fragilis* were grown anaerobically in DMM supplemented with 6.6 mM (closed symbols) or 0.0066 mM (open symbols) of KH_2PO_4. Growth was measured at OD_{600}. Data presented are the means ± standard deviations of triplicate cultures. **Significantly different from wild type ($p<0.01$), *Significantly different from wild type ($p<0.05$).
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polysaccharide biosynthesis loci B (PS B, BF1828-BF1848) and E (PS E, BF2566-BF2586), or internal fragment of BF3397 were amplified by PCR. The amplicons were purified using a QIAquick Gel Extraction Kit (Qiagen) after agarose gel separation and used as bait DNA. After incubation, the samples were subjected to electrophoresis on a 5% polyacrylamide gel and visualized using the SYBR Green I Nucleic Acid Gel Stain Kit (Invitrogen).

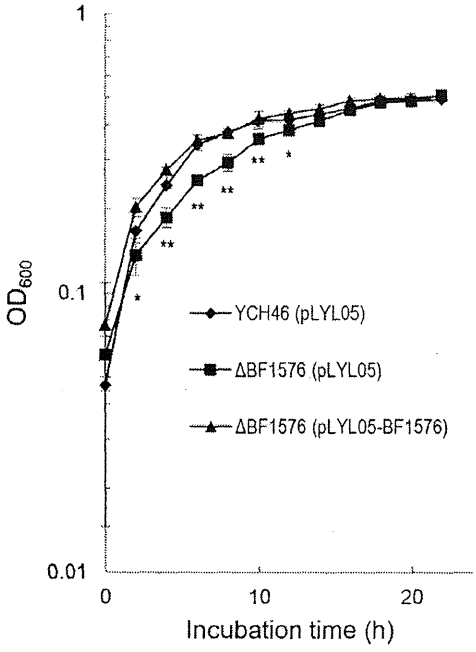

Figure 3. Repletion of BF1576 by plasmid complementation. *B. fragilis* wild type strains harboring pLYL05-Exp and the ΔBF1576 strain harboring pLYL05-Exp or pLYL1576 were grown anaerobically in a limiting amount of Pi (0.0066 mM of KH_2PO_4) in DMM supplemented with 50 μg/ml of cefoxitin. Growth was measured at OD_{600}. Data were calculated from triplicate cultures and expressed as means ± standard deviations. **Significantly different from the ΔBF1576 strain harboring pLYL05-Exp ($p<0.01$), *Significantly different from the ΔBF1576 strain harboring pLYL05-Exp ($p<0.05$).
doi:10.1371/journal.pone.0053829.g003

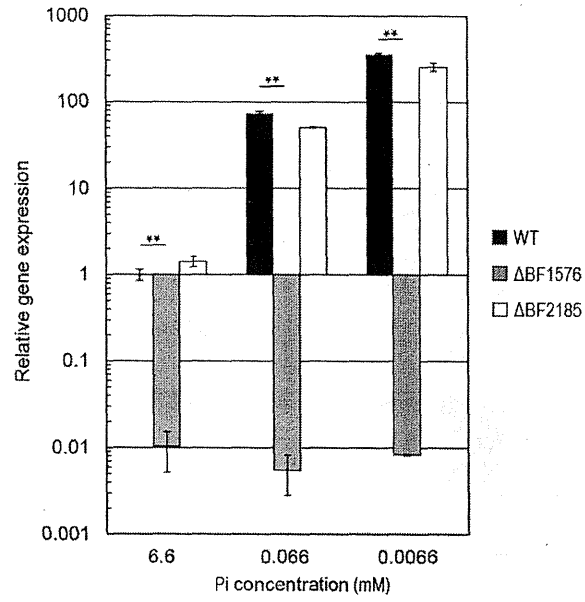

Figure 4. Induction of *pstC* expression in response to Pi limitation. Wild type, ΔBF1576, and ΔBF2185 strains of *B. fragilis* were cultured in DMM supplemented with varying concentrations of Pi as indicated. Total RNA was extracted from mid-logarithmic phase cultures, and *pstC* expression levels were compared by qPCR. The transcriptional level of *pstC* was normalized with that of *rpoD* and shown relative to that of a wild type strain in DMM supplemented with 6.6 mM KH_2PO_4 (Pi-rich media). Black column: wild type; gray column: ΔBF1576; white column: ΔBF2185. **Significantly different from the wild type strain ($p<0.01$).
doi:10.1371/journal.pone.0053829.g004

Peritoneal Abscess Model

Five male C57BL/6J Jcl mice (7 weeks old) were injected intraperitoneally with 0.2 ml of an inoculum (2.0×10^8 colony-forming unit each) that was prepared by mixing *B. fragilis* cell suspensions (wild type, ΔBF1576, ΔBF1576 complemented with pLYL1576, or a combination of wild type and ΔBF1576) with the supernatant of an autoclaved rat fecal suspension at a 1:1 (vol/vol) ratio. The autoclaved rat fecal suspension did not induce abscesses when injected alone into the mouse peritoneal cavity, and was used as an adjuvant for abscess formation. Mice were sacrificed at 3, 7, or 14 days after challenge, and abscess formation was evaluated. The incidence of peritoneal abscess, the number of abscesses and surviving *B. fragilis* were compared. Prior to dissection of the peritoneum, 10 ml of PBS (pH7.4) was injected into the peritoneal cavity and then recovered. The number of infiltrated inflammatory cells in the recovered fluid was counted using a hemocytometer. The surviving cell number inside the peritoneal abscesse was determined as follows. The collected abscesses were weighed and homogenized in 10 volumes of autoclaved PBS (pH7.4) using Teflon glinder. Serial dilutions were made with PBS (pH7.4) and the appropriate dilutions were spread onto GAM agar plate. The plates were incubated anaerobically at 37°C for 48 h and the numbers of colony grown on the plates were counted. Wild type *B. fragilis* and the ΔBF1576 mutant were discriminated using colony PCR with a primer pair encompassing the deletion site in ΔBF1576 (Table S1), when necessary. At least, 96 colonies per abscess were screened.

Competitive Intestinal Colonization Model

Three male germ-free BALB/c mice (8 weeks old) were orally inoculated by gavage with the mixture of wild type and ΔBF1576 *B. fragilis* strain (2.0×10^8 colony-forming unit each). Feces were collected periodically (7 and 14 days after challenge), and appropriate dilutions of the fecal suspensions were spread on GAM agar plates. Colony PCR was performed with the primer pair encompassing the deletion site in ΔBF1576 to compare their population levels of wild and the mutant *B. fragilis* strains in the mouse intestines. In this experiment, mice were kept in a vinyl isolator to maintain the gnotobiotic status.

Statistical Analysis

The growth rates, qPCR data and the number of abscess were statistically analyzed with Student's *t* test. The incidence of peritoneal abscess and wild-type/*phoB* mutant ratios in the intestines and abscesses were analyzed with Fisher's exact test and Chi-square test, respectively. Changes were considered to be significantly different when the *p*-values were less than 0.05.

Microarray Accession Numbers

Microarray data have been deposited in the Gene Expression Omnibus database (www.ncbi.hlm.nih.gov/projects/geo) under the following accession numbers: normalized data, GSE27439; platform, GPL13213; and raw data files, GSM678272 to GSM678275.

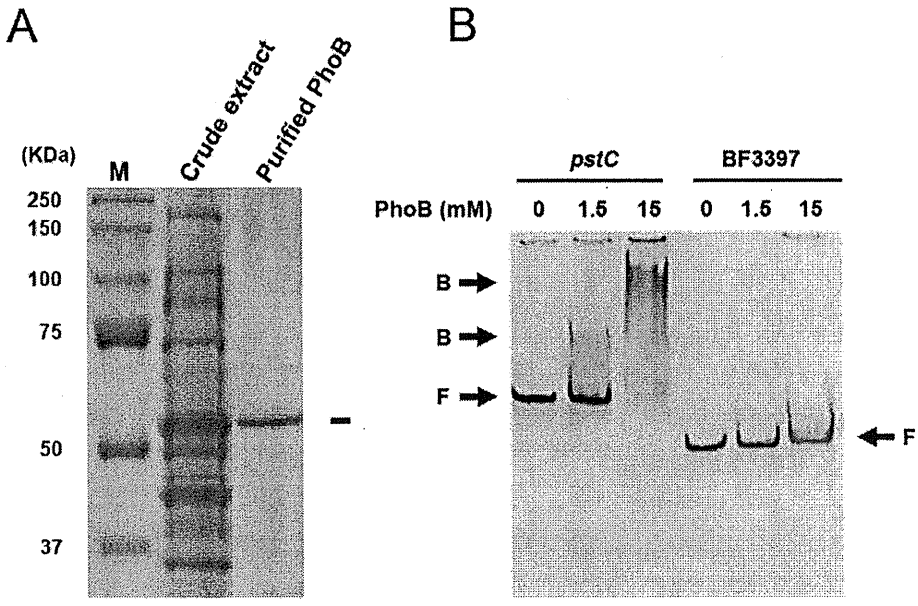

Figure 5. PhoB binds to *pstC* promoter. (A) Expression and purification of recombinant *B. fragilis* PhoB in *E. coli*. M, molecular size markers. (B) Binding of PhoB to *pstC* promoter. Recombinant PhoB (1.5 or 15 μM of final concentration) was mixed with the amplified promoter region of *pstC* or internal region of BF3397, and their interaction was evaluated by electrophoretic motility shift assay. Zero denotes that no protein was added to the reaction mixture. B: PhoB-bound probes; F: free probes.
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Ethics

All animal experiments were performed according to the guidelines for animal experiments at the University of Tokushima. The experimental design was approved by the Animal Experiment Committee of the University of Tokushima.

Results

Identification of a PhoRB System in *B. fragilis*

The genome of sequenced *B. fragilis* strain YCH46 contains 70 two-component signal transduction systems (TCS), which include orphan kinases and response regulators (Table S2). A BLAST search was performed to identify a TCS of *B. fragilis* that corresponds to PhoRB in other bacteria. Amino acid sequences of *E. coli* PhoR or PhoB were used as queries against the *B. fragilis* genome. The proteins encoded by BF1575 and BF1576 showed the best similarities to *E. coli* PhoR and PhoB, respectively (39% and 29% identity over 50% alignment length, respectively). TCS proteins encoded by BF2185 and BF2186 showed the second highest homologies to PhoRB of *E. coli*. PhoB regulates the expression of a phosphate-specific transport (Pst) system to acquire Pi [3]. The Pst system is composed of five components encoded within the *pstSCAB-phoU* operon. According to the gene annotation, in the *B. fragilis pst* operon, *pstS* is located just upstream of the *pstCAB-phoU* operon (BF2756-BF2753) in a head-to-head manner.

B. fragilis strain YCH46 was grown in DMM supplemented with varying concentrations of KH_2PO_4 (final concentration of 6.6, 0.066, or 0.0066 mM). As shown in Figure 1A, a decrease in Pi from 6.6 mM to 0.066 mM did not result in the growth retardation (the doubling times; 2.14±0.08 h vs 1.93±0.03 h). Clear growth retardation was observed when the Pi concentration was limited to 0.0066 mM (the doubling time; 3.85±0.50 h). Based on this result, growth media supplemented with 0.0066 mM KH_2PO_4 was used as the Pi-limiting condition in subsequent experiments. RNA was extracted from mid-logarithmic phase cultures and qPCR was performed for BF1575, BF1576, and *pstC* (BF2756). As shown in Figure 1B, the expression levels of these genes were negatively correlated with Pi level and were elevated over 10 fold compared with those assayed in Pi-rich media (6.6 mM of KH_2PO_4).

BF1576 was disrupted (ΔBF1576) to determine the role of the *phoB* homolog in adaptation to Pi limitation (Figure 2A). BF2185 encodes a protein with the second highest homology to *E. coli* PhoB. A BF2185 deletion mutant (ΔBF2185) was constructed for comparison to ΔBF1576. Growth of the ΔBF1576 strain was similar in Pi-rich media (the doubling time; 2.31±0.02 h) but slower than that of the wild type and ΔBF2185 strains when they were cultured in Pi-limited media (0.0066 mM of KH_2PO_4) (Figure 2B); the doubling times of ΔBF1576, wild type and ΔBF2185 were 5.88±1.21 h, 3.85±0.50 h and 4.12±0.46 h, respectively. Complementation of the ΔBF1576 strain with a plasmid harboring BF1576 restored the growth of this mutant (the doubling time; 4.26±0.40 h) to wild type level in DMM supplemented with 0.0066 mM of KH_2PO_4 (Figure 3). Under Pi limitation, PhoB binds the "Pho box" regulatory element in the promoter of the *pst* operon and activates expression of the *pst* genes. A comparison of the expression of *pstC* in the wild type and mutant strains in low Pi revealed that the ΔBF1576 strain showed a trace amount of *pstC* expression while the expression level in the ΔBF2185 strain was equivalent to that of the wild type strain (Figure 4). These results indicated that BF1576 encodes PhoB in *B. fragilis*.

To confirm the direct association of PhoB with *pstC* expression, the protein was overexpressed in *E. coli* and purified (Figure 5A). The binding of the recombinant PhoB to the *pstC* promoter was assessed by electrophoretic motility shift assay (EMSA). EMSA demonstrated the direct binding of PhoB to the promoter of *pst*

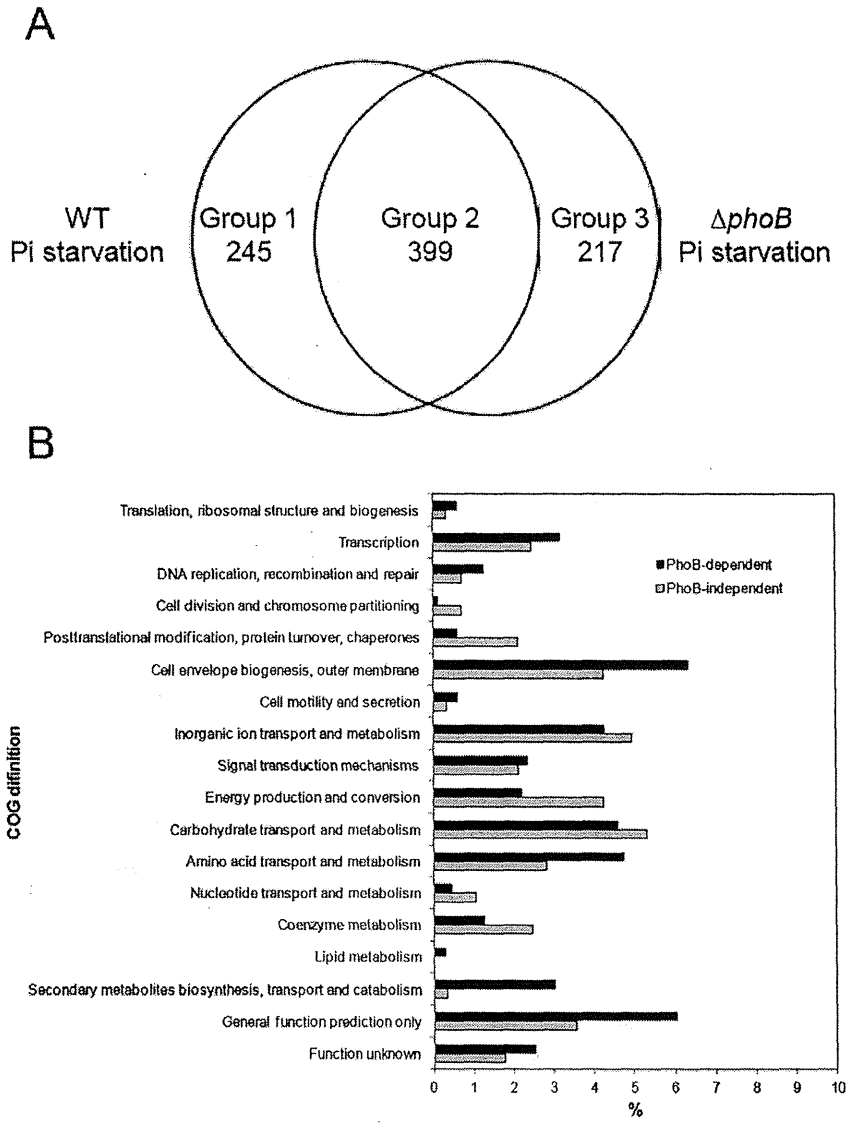

Figure 6. PhoB-dependent genes in *B. fragilis*. (A) Classification of genes whose expression levels were altered more than 4-fold under Pi limitation (0.0066 mM of KH_2PO_4) in wild type and Δ*phoB* mutant strains, compared to levels in wild type in the presence of sufficient Pi (6.6 mM of KH_2PO_4). Left and right circles indicate the differentially expressed genes of wild type and Δ*phoB* mutant strains with expressions that were altered over 4-fold under Pi limitation. Group 1: includes 245 PhoB-dependent Pi-response genes. Group 3:217 PhoB-dependent genes unrelated to Pi response. Group 2 contains two subgroups. Group 2a: 276 PhoB-independent Pi-response genes (fold change in expression in response to Pi-limitation was similar between wild type and Δ*phoB* mutant strains); Group 2b: 123 PhoB-dependent Pi response genes (the extent of change in expression in response to Pi-limitation was different >2-fold between wild type and Δ*phoB* mutant strains). (B) COG classification of PhoB-dependent (black bar) and PhoB-independent (white bar) genes that are differentially expressed during Pi starvation. The percentage of genes classified into each COG category is relative to the total number of genes in each group. Fifty-five percent of the PhoB-dependent (585 genes) and 60% of the PhoB-independent (276 genes) groups could not be assigned to a COG category (that data are not included in this figure).
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operon (512-bp fragment) but not to another DNA fragment used as a control (290-bp internal fragment of BF3397) (Figure 5B).

Pho Regulon in *B. fragilis*

Transcriptome analysis was performed to identify the regulatory circuit of PhoB in *B. fragilis*. Compared to the wild type expression profile in Pi-rich media (6.6 mM of KH_2PO_4), the expression of 644 (wild type) and 616 (Δ*phoB* mutant) genes changed more than 4-fold under Pi-limiting conditions (Figure 6A). Of these, the expression levels of 399 genes were altered in both wild-type and the Δ*phoB* mutant (group 2 in Figure 6A), although some of the genes underwent different degree of transcriptional changes depending on the strain (e.g., BF2091 and BF2391 in Table 2). Group 1 in Figure 6A included 245 genes with expression levels that changed more than 4-fold under Pi-limitation (6.6 mM to 0.0066 mM of KH_2PO_4) in wild type, but did not change in the Δ*phoB* mutant, which corresponded to PhoB-dependent Pi

Table 2. Representative list of differentially expressed genes in wild type and Δ*phoB B. fragilis* strains in Pi-limiting conditions.

Gene	Function	Fold change [a]		Group [b]
		WT	Δ*phoB*	
Inorganic ion metabolism and transporter				
BF0375	polyphosphate-selective porin O	+3.55	−2.70	2b
BF0480	alkaline phosphatase III precursor	+6.37	–	1
BF0578	potassium-transporting ATPase subunit A	+121	+161	2a
BF0579	K+-transporting ATPase B chain	+151	+238	2a
BF0580	K+-transporting ATPase C chain	+163	+261	2a
BF0582	osmosensitive K+ channel histidine kinase	+88.5	+128	2a
BF1417	putative ferrous iron transport protein	–	−8.18	3
BF1756	alkaline phosphatase	+12.2	–	1
BF2091	cation efflux system protein	+22.3	−4.69	2b
BF2092	CzcA family cation efflux system protein	+22.4	−5.67	2b
BF2289	putative polyphosphate-selective porin O	−3.16	+1.37	2b
BF2392	putative cation efflux pump	+80.0	+21.7	2b
BF2393	putative cation efflux pump	+61.7	+17.1	2b
BF2604	multidrug efflux pump BexA	−4.76	–	1
BF2645	polyphosphate kinase	+24.4	–	1
BF2646	putative exopolyphosphatase	+42.1	–	1
BF2753 (*phoU*)	putative transcriptional regulator for phosphate uptake	–	−16.9	3
BF2754 (*pstB*)	putative phosphate transport ATP-binding protein	–	−353	3
BF2755 (*pstA*)	putative ABC transporter permease protein	–	−542	3
BF2756 (*pstC*)	putative ABC transporter permease protein	–	−428	3
BF2757 (*pstS*)	phosphate ABC transporter phosphate-binding protein	+4.58	−103	2b
BF3145	multidrug resistance ABC transporter	+4.32	–	1
BF3309	sugar transporter	+17.7	+15.5	2a
BF3350	putative glucose/galactose transporter	+13.3	+5.43	2b
BF3714	polyphosphate kinase	+5.22	+4.94	2a
BF3715	putative phosphate/sulphate permeases	–	−52.2	3
BF3721	cation efflux system protein	–	−5.76	3
BF3722	AcrB/AcrD/AcrF family cation efflux system protein	–	−5.69	3
BF4130	putative phosphate ABC transporter phosphate-binding component	+4.10	–	1
BF4134	MotA/TolQ/ExbB proton channel	+3.78	−1.42	2b
BF4348	putative calcium-transporting ATPase	−2.42	+1.68	2b
BF4351	putative Na+-dependent phosphate transporter	−101	–	1
BF4541	acid phosphatase	+328	–	1
Nucleic acid repair, recombination and metabolism				
BF1683	Holliday junction resolvase-like protein	–	−4.15	3
BF1862	SOS mutagenesis and repair protein UmuC	+7.84	–	1
BF1863	error-prone repair: SOS-response transcriptional repressor UmuD	+11.2	–	1
BF1883	putative AAA family ATPase	+4.42	–	1
BF2939	2′,3′-cyclic nucleotide 2′-phosphodiesterase	–	−4.37	3
BF3503	uracil-DNA glycosylase	+11.0	–	1
BF4422	DNA-binding protein HU-beta	–	−5.00	3
Protein repair and chaperons				
BF1205	endopeptidase Clp ATP-binding chain B	+9.42	+6.60	2a
BF1225	molecular chaperone DnaK	+12.5	+7.93	2a
BF1742	chaperone protein DnaJ	+6.75	+4.06	2a
BF1743	GrpE protein	+8.08	+4.23	2a
BF2021	peptidyl-prolyl cis-trans isomerase	−5.05	–	1
BF2409	heat shock protein 90	+16.5	+8.23	2b

Table 2. Cont.

Gene	Function	Fold change [a]		Group [b]
		WT	Δ*phoB*	
BF2625	small heat shock protein	+7.71	–	1
BF3377	putative chaperone DnaJ	+13.3	+5.01	2b
BF3395	chaperonin GroEL	+8.23	+7.10	2a
BF3396	co-chaperonin GroES	+19.3	+12.1	2a
Cell envelope biogenesis and outer membrane				
BF0002	putative outer membrane protein TolC	−1.45	+3.39	2b
BF1428 (*upaY*)	transcriptional regulatory protein UpxY homolog in capsular polysaccharide synthesis locus (PSA)	−6.50	−16.4	2b
BF1828 (*upbY*)	transcriptional regulatory protein UpxY homolog in capsular polysaccharide synthesis locus (PSB)	−5.55	+10.0	2b
BF2391	outer membrane efflux protein oprM precursor	+106	+28.4	2b
BF2585 (*upeY*)	transcriptional regulatory protein UpxY homolog in capsular polysaccharide synthesis locus (PSE)	–	−330	3

[a]Fold change (relative to wild-type 6.6 mM Pi) was determined from the microarray expression data as described in the Materials and Methods.
[b]The definition of each group is described in legend to Figure 6.
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response genes. In contrast, Group 3 included 217 genes whose expression levels in the Δ*phoB* mutant were affected more than 4-fold under Pi limitation but were unchanged in the wild type strain, indicating that they were PhoB-dependent genes that were unrelated to the Pi response. The genes classified as Group 2 were divided into two subgroups. Group 2a contained 276 PhoB-independent Pi-response genes (the magnitude of changed expression levels in response to Pi-limitation was similar between the wild type and Δ*phoB* mutant strains) and Group 2b had 123 PhoB-dependent Pi response genes (the extent of change in expression in response to Pi-limitation was different >2-fold between wild type and Δ*phoB* mutant strains). The 585 genes classified into Groups 1, 2b, and 3 were defined as Pho-regulon genes. Representative genes with significantly altered expression patterns under Pi limitation are listed in Table 2. Of these, PhoB-dependent genes included those associated with Pi metabolism such as PstSCAB, alkaline phosphatase (BF0480 and BF1756), polyphosphate kinase (BF2645), exopolyphosphatase (BF2646), a putative phosphate/sulphate permease (BF3715) and acid phosphatase (BF4541). In addition to Pi metabolism, genes involved in capsular polysaccharide biosynthesis (PS A, B and E), ferrous iron transport (BF1417), protein repair and chaperon activity, and DNA repair and recombination were also regulated in a PhoB-dependent manner. These data suggest that PhoB may be involved in *B. fragilis* virulence, the stress response and iron homeostasis, as well as in Pi metabolism. To assess the effect of KH_2PO_4 limitation on pH of the culture media, we checked the pH of 12-h and 24-h culture of wild or *phoB* mutant strain. The pH of these cultures were kept around 7.6, indicating the effect of acid stress could be ruled out (data not shown). The differentially expressed genes during Pi starvation were assigned to the Cluster of orthologous gene (COG) classification (Figure 6B). The PhoB-regulated function preferentially distributed into Transcription; DNA replication, recombination and repair; Cell envelope biogenesis, outer membrane; Amino acid transport and metabolism; and Secondary metabolites biogenesis, transport and catabolism.

To verify the microarray analysis data, qPCR was performed on the representative PhoB regulon genes listed in Table 2, which include stress-response, phosphate metabolism and polysaccharide biosynthesis (PS B and PS E). As shown in Figure 7, the expression profiles correlated well the results from microarray analysis (Table 2).

Role of PhoB in Peritoneal Abscess Formation

Wild type and Δ*phoB* mutant *B. fragilis* strains were inoculated into the peritoneal cavities of C57BL/6J mice to compare their ability to induce abscess formation. Mice were sacrificed at 3, 7, or 14 days after challenge, and the number of abscesses was counted. One week after challenge, the wild type *B. fragilis* strain formed peritoneal abscesses in 80% of the mice (Table 3). Abscesses were observed in only 40% of the mice tested with the Δ*phoB* mutant. In addition, a total of 13 abscesses were produced by wild type *B. fragilis*, while only 2 abscesses were observed with the Δ*phoB* mutant. Complementation of the Δ*phoB* mutant with a plasmid harboring *phoB* restored the number of abscesses. However, during the early stage of peritoneal infection (3 days after challenge), no difference was found in incidence and abscess number between wild type and Δ*phoB* mutants. The viable cell numbers inside the abscesses were also not significantly different among the strans (Table 4). In addition, inflammatory cell infiltration into peritoneal cavity was similar between the wild type strain ($6.20\pm2.96\times10^6$/ml of the recovered fluid) and *phoB* mutant ($5.73\pm2.83\times10^6$/ml) at an early stage (3 days) of infection ($2.92\pm1.10\times10^6$/ml in autoclaved rat feces alone). These findings indicate that in *B. fragilis*, PhoB plays a role in abscess persistence rather than formation.

To compare the growth and survival of wild type and Δ*phoB B. fragilis* strains under various conditions, these strains were equally mixed and inoculated into GAM broth, mice intestines or the peritoneal cavity. After periodical sampling from liquid culture, feces and abscesses, samples were spread on GAM agar plates, and the population levels of wild type and Δ*phoB B. fragilis* strains were evaluated by discriminating colony PCR. Deletion of *phoB* did not affect growth in rich media (GAM broth), and the ratios of wild type to *phoB* mutant strains were 0.67 to 1.07 until 72-h cultivation (Figure 8A). The *phoB* deletion also had no effect on intestinal colonization, and equivalent populations of each cell type were maintained until two weeks after inoculation into the gut of eight-

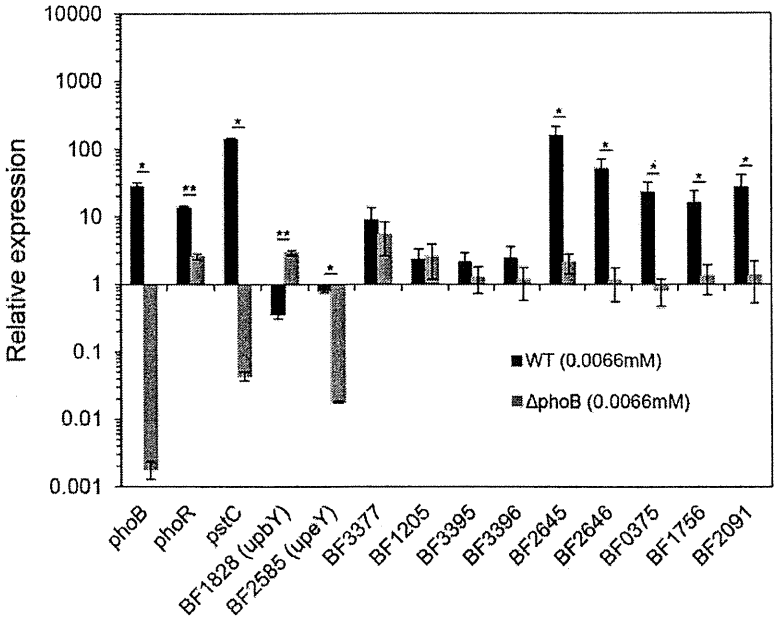

Figure 7. qPCR verification of differentially expressed PhoB-dependent genes identified by microarray analysis. The transcriptional levels of representative genes in wild type (closed column) and *phoB* deletion mutant (open column) under Pi limitation (0.0066 mM of KH_2PO_4) is shown in relation to those in wild type under Pi rich condition (6.6 mM of KH_2PO_4). Annotation of each gene is as listed in Table 2: BF1576 (PhoB), BF1575 (PhoR), BF2185 (PstC), BF1828 (UpbY, product of the first gene product in PS B), BF2585 (UpbY, product of the first gene product in PS E), BF3377 (DnaJ), BF1205 (endopeptidase Clp), BF3395 (GroEL), BF3396 (GroES), BF2645 (polyphoaphate kinase), BF1576 (alkaline phosphatase), and BF2091 (cation efflux system protein). **Significantly different from the wild type strain, *Significantly different from the wild type strain ($p<0.05$).
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week-old male BALB/c germ-free mice (Figure 8B). In contrast, the number of Δ*phoB* mutant cells decreased more rapidly than the number of wild type cells when survival within the peritoneal abscess was compared. The ratio of Δ*phoB* mutant to wild type cells inside the abscess was reduced to one-third at two weeks after intra-peritoneal inoculation (Figure 8B). The ratios of wild type to *phoB* mutant after one and two week inoculation were significantly different (Chi-squre p values are 0.0173 and less than 0.0001, respectively). These results indicate that PhoB is involved in the survival of *B. fragilis* in stressful extraintestinal environments.

Table 3. Peritoneal abscess formation by *B. fragilis* strains.

B. fragilis strain	Abscess formation [a]					
	Incidence			Number of abscess[b]		
	3 days	7 days	14 days	3 days	7 days	14 days
YCH46 (wild type)	5/5	4/5	4/5	3.40±1.34	2.00±1.22	1.40±1.14
ΔBF1576 (ΔphoB)	5/5	2/5*	2/5*	3.00±1.58	0.40±0.55*	0.40±0.55
ΔBF1576 (pLYL1576)	5/5	4/5	4/5	2.20±0.45	1.60±1.52	0.80±0.45

[a]Five C57BL/6J mice were intraperitoneally challenged with *B. fragilis* strains. Mice were sacrificed and abscess formation was evaluated on the indicated days after challenge.
[b]Number of abscess in each mouse (mean ± SD) is shown.
*Significant difference from YCH46 (wild type), $p<0.05$.
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Discussion

Microbes inhabit all of the environmentally exposed anatomical sites of the human body. Individual microbial species have adapted uniquely to their preferential niches. Signals present in these microenvironments play key roles in shaping host-microbe interactions. Through these signals, commensal bacteria provide beneficial actions such as digestion of otherwise undigestible nutrients, synthesis of essential vitamins and competitive colonization resistance to foreign pathogenic bacteria (symbiosis). Disruption of these homeostatic signals leads to pathogenic conditions such as overgrowth or submucosal invasion of commensal bacteria, which elicit the host inflammatory response (dysbiosis). The persistence of dysbiosis results in diseases such as inflammatory bowel syndrome [23,24], cancer [25] and obesity [26–29].

Table 4. Viable *B. fragilis* cell numbers in the peritoneal abscesses [a].

Strains	Viable cell counts (log_{10} cfu/abscess) [b]		
	3 days	7 days	14 days
YCH46 (wild type)	6.58±1.12	6.21±1.08	6.24±1.64
phoB mutant	6.54±1.14	6.50±0.81	5.91±0.23

[a]Each of the strains (ca. 2.0×10^8 cells) was individually inoculated into the mouse peritoneal cavity to form abscesses. Each of the corrected abscesses was homogenized in PBS, and the serial dilutions were plated on GAM agar plates.
[b]Data were mean ± SD. No significant differences were observed.
doi:10.1371/journal.pone.0053829.t004

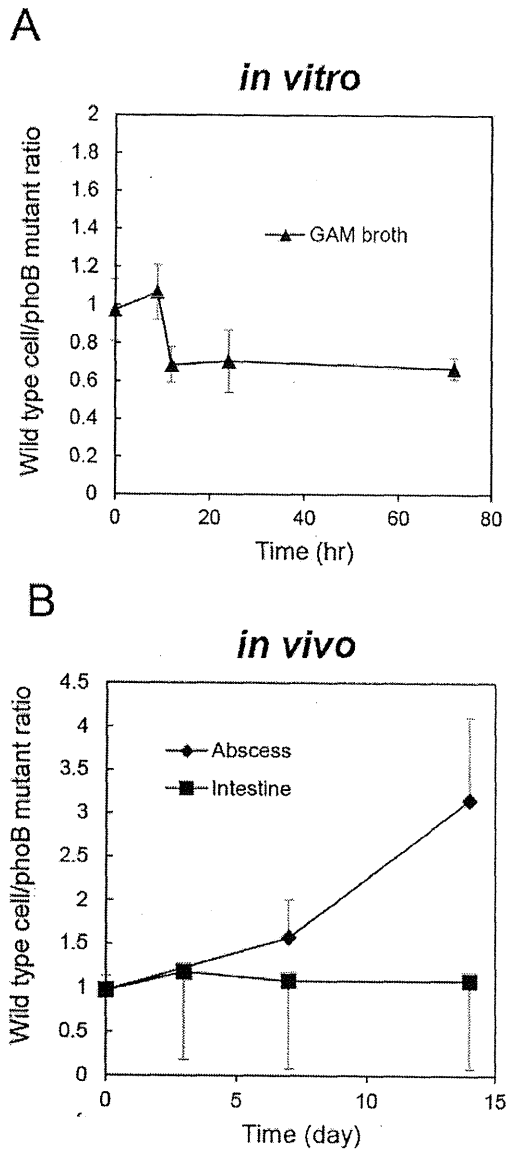

Figure 8. Competitive growth assay of wild type and Δ*phoB B. fragilis* strains *in vitro* (A) and *in vivo* (B). These strains were equally mixed and inoculated into GAM broth, mice intestines or the peritoneal cavity. After periodical sampling from liquid culture, feces and abscesses, samples were spread on GAM agar plates, and the population levels of wild type and Δ*phoB B. fragilis* strains were evaluated by discriminating colony PCR. The data were expressed as wild type/Δ*phoB* mutant ratios. The ratios of wild type and *phoB* mutant strains in the intestine and abscess were compared by Chi-square test.
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The intestinal tract harbors the most densely populated microbiota in the human body. *Bacteroides* is one of the predominant groups of gut commensals [10]. Although *B. fragilis* is one of the most pathogenic species of the genus *Bacteroides* and often causes peritonitis, intra-abdominal abscesses and septicemia [14–16], the bacterium is considered to be a keystone microbe that controls the microbial ecosystem in the gut [30]. The two-component signal transduction system (TCS) is a major bacterial environmental sensing system. The whole genome sequence of *B. fragilis* revealed that this anaerobe possesses 70 TCS [31]. They undoubtedly play essential roles in the processing of environmental signals within the gut. However, the environmental signals that induce the TCS are largely unknown. Pi limitation is a well characterized environmental signal sensed by PhoRB, a TCS widely distributed among prokaryotes. Pi depletion after abdominal surgical operations is a risk factor for sepsis caused by gut-derived opportunistic pathogens [11,12]. In this study, we focused on this stimulus (Pi concentration) to assess the importance of environmental sensing systems on shaping the symbiosis and dysbiosis within the gut.

We identified the PhoRB system in the sequenced *B. fragilis* strain YCH46 and demonstrated that PhoB, as in other bacteria, plays a role in adaptation to Pi-limiting conditions. However, it is unlikely that PhoRB is the sole Pi acquisition system in *B. fragilis* because *phoB* deletion did not result in a complete growth defect (Figure 1A). No difference was observed in the maximum optical density of the culture in Pi-limiting media. In addition to the *pst* operon, PhoB regulates the expression of 585 genes in *B. fragilis*. Of these, 217 are regulated in a Pi-dependent manner (Group 1 in Figure 6A). The remaining 368 genes are regulated independent of Pi concentration (Group 2b and Group 3 in Figure 6A). This group includes several stress response genes and capsular polysaccharide biosynthetic genes (PS A, B and PS E).

Capsular polysaccharides are a major virulence factor of *B. fragilis* and play an essential role in abscess formation. *B. fragilis* produces as many as nine capsular polysaccharides [31], and the seven of them phase vary via the inversion of promoters locate upstream the PS loci [32]. Of these, abscess forming capsular polysaccharides are only two types of capsular polysaccharides, PS A and PS B, in strain NCTC9343 [14,15]. The remaining seven capsule types are not essentially involved in abscess formation. Interestingly, the expression of these PS loci in strain YCH46 is regulated by PhoB: PhoB is positively associated with PS E expression but negatively with PS B expression. These regulatory activities might be indirect since PhoB did not bind these PS promoters in the EMSA assay (data not shown). However, as reported by Chatzidaki-Livanis *et al.*, the transcription of each of the phase variable PS loci is not simply dependent on the promoter orientations but regulated in a complex manner, in which the UpxZ proteins that encoded by the second gene of each of the phase variable PS loci inhibit the synthesis of heterologous capsular polysaccharides [33]. It still remains the possibility that the *B. fragilis* PhoB bind to the PS promoters other than those of PS B and PS E, affecting the expression levels of these PS loci. Although *phoB* deletion reduced the transcriptional level of the PS A locus, the role of PhoB on abscessogenic PS A production is likely to be weak since the difference in the transcriptional levels of many genes in the PS A locus was small between wild type and Δ*phoB* mutant strains (Tables S3 to S6). Due to the heterogeneity of PS loci among *B. fragilis* as strains NCTC9343 and YCH46 share only the PS B locus [34], it is difficult to predict the physiological significance of PhoB regulation on polysaccharide biosynthesis in strain YCH46. Although the dose-dependency should be observed to compare the abscessogenic potential between the strains, our results in the mouse peritoneal abscess model indicate that these PhoB-regulated polysaccharides in strain YCH46 are not essential for abscess formation. Rather, these polysaccharides (PS B and PS E) and other Pho regulon genes are probably involved in survival of *B. fragilis* at extraintestinal sites such as the peritoneal cavity and the interior of abscesses.

PhoB has been associated with virulence in several pathogenic bacteria [3]. In many cases, the role of PhoB on virulence has been

evaluated by the use of a constitutively active model obtained from mutation of the Pst system, which negatively regulates the Pho regulon, regardless of Pi concentration. In this model, PhoB activation reduced toxin production and pillus formation in *Vibrio cholerae* [35], and suppressed the growth of avian pathogenic *E. coli* in the intestinal tract [36]. However, Pho regulon genes have also been induced during infection of models with *Yersinia pestis*, *Erwinia chrysanthemi*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* [4–8]. These findings indicate that the Pho regulon may work at particular stages of the infection process and that timely activation of this regulon is essential for adaptation. Therefore, constitutively active PhoB might interfere with the infection process.

In this study, it was concluded that PhoB does not directly affect the abscess formation of *B. fragilis* when virulence was assessed using a mouse model. The results also showed that the inflammatory cell infiltrations into peritoneal cavity by wild and Δ*phoB B. fragilis* strains were similar at an early stage (3 days) of infection. In addition, the viable cell number/abscess of both strains was similar when each strain was individually inoculated into peritoneal cavity (Table 4). On the other hand, the *phoB* deletion resulted in the faster clearance of the cells from abscess than wild strain in the mixed inoculation experiments, while the number of *phoB* mutant was similar to that of wild type strain in the GAM broth or mouse intestinal tract. However, it is possible that the mutant could not compete with the wild type for intestinal colonization under special conditions such as nutrient restriction and mucosal inflammation. These results indicate that PhoB regulates the stress adaptation of *B. fragilis* and contributes to survival of this anaerobe inside peritoneal abscesses. It is also possible that the increase of wild type to *phoB* mutant ratios in the abscess might only represent the difference in their growth rates. The monitoring of growth status of both strains and Pi-levels in the peritoneal abscess is necessary in the future study to determine the role of the PhoB in *B. fragilis*.

Bacteroides species are the causative agents of post-operative abdominal infections. Oral Pi administration prevents translocation of *P. aeruginosa* after surgical operations [11]. Signal transduction via the PhoRB system is a potential target for control of intra-abdominal infection by *B. fragilis*. The results presented here provide information that may be used to develop novel strategies for the handling of intra-abdominal infections to improve the recovery.

Supporting Information

Table S1 PCR primers used in this study.
(XLSX)

Table S2 Two-component signal transduction systems (TCS) in *B. fragilis* strain YCH46.
(XLSX)

Table S3 The wid-type genes whose expressions were induced >4-fold by Pi starvation.
(XLSX)

Table S4 The wid-type genes whose expressions were repressed >4-fold by Pi starvation.
(XLSX)

Table S5 The *phoB* mutant genes whose expressions were induced >4-fold by Pi starvation.
(XLSX)

Table S6 The *phoB* mutant genes whose expressions were repressed >4-fold by Pi starvation.
(XLSX)

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Author Contributions

Conceived and designed the experiments: SW HNI TH KY TK. Performed the experiments: SW HNI MI TK. Analyzed the data: SW HNI HH TH TK. Contributed reagents/materials/analysis tools: HH HM. Wrote the paper: SW TK.

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ORIGINAL ARTICLE

Survey of rituximab treatment for childhood-onset refractory nephrotic syndrome

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Abstract
Background Rituximab (RTX) is a promising option for treating childhood-onset steroid-dependent (SDNS), frequently relapsing (FRNS), and steroid-resistant (SRNS) nephrotic syndrome.
Methods We retrospectively surveyed RTX treatment for these conditions to evaluate its indications, efficacy and adverse events. Questionnaires were sent to 141 hospitals in Japan.
Results Seventy-four patients (52 SDNS; 3 FRNS; 19 SRNS) were treated with RTX because of resistance to various immunosuppressive agents. Most patients received a single administration of RTX (85%). Forty-one of 53 SDNS/FRNS (77%) and 5 of 17 SRNS (29%) patients successfully discontinued prednisolone (16 SDNS/FRNS and 6 SRNS achieved their first discontinuation since onset), and 17 out of 53 SDNS/FRNS patients (31%) discontinued cyclosporine. However, 28 of the 53 patients (51%) relapsed. Although immunosuppressive agents did not extend B cell depletion, relapses were significantly less if immunosuppressive agents were continued after RTX (P=0.006; hazard ratio=0.2). Among the SRNS patients, complete (n=6) and partial remission (n=6) were achieved. No life-threatening adverse events were experienced.
Conclusions Although this was a multi-center survey where treatment of nephrotic syndrome varied between centers, the steroid-sparing effect of RTX in SDNS/FRNS was excellent. If single administration of RTX is chosen, continuation of immunosuppressive agents is recommended for prevention of relapse.

Keywords Rituximab · Nephrotic syndrome · Children · Steroid · Steroid-dependent nephrotic syndrome · Steroid-resistant nephrotic syndrome · Immunosuppressive agents

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Introduction

Treatment of refractory childhood nephrotic syndrome, such as frequently relapsing nephrotic syndrome (FRNS), steroid-dependent nephrotic syndrome (SDNS), and steroid-resistant nephrotic syndrome (SRNS), is still challenging. Although various immunosuppressive agents are effective for these conditions, in substantial numbers of children it remains intractable.

Most such patients suffer serious adverse effects of steroid and immunosuppressive agents. Recently, rituximab (RTX), a monoclonal antibody targeting the B cell-specific antigen CD20, has been proven to be effective for FRNS/SDNS and SRNS in children [1–8].

The incidental discovery of the effects of this drug has improved the prognosis of childhood SDNS and SRNS [9,

10]. Although RTX is still off-label and its safety for use in nephrotic syndrome has not yet been proven, pediatric nephrologists have started to use RTX in many countries. We previously reported a prospective study of single-dose therapy with RTX for 12 children with intractable SDNS [3]. All patients were able to discontinue steroids after RTX administration. We also found a significant decrease in the frequency of relapses, period of steroid use and mean steroid dosages after RTX treatment. However, the efficacy of the single-dose treatment was not always long-lasting, and the subsequent recovery of B cells in the peripheral blood sometimes resulted in disease recurrence. Therefore, maintenance therapy with some immunosuppressive agents after a single dose of RTX may be a good option to lengthen remission [11, 12]. Although RTX is relatively well tolerated, some severe or life-threatening adverse events, including progressive multifocal leukoencephalopathy [13], interstitial pneumonia [14], and ulcerative colitis [15], have been anecdotally reported. Such severe life-threatening adverse events are rarely experienced with existing immunosuppressive agents, including cyclosporine (CsA), cyclophosphamide, mycophenolate mofetil, tacrolimus, and mizoribine. We conducted a national survey of RTX use for refractory nephrotic syndrome to investigate its indications, efficacy, and adverse events.

Patients and methods

Patients

Questionnaires regarding the use of RTX against childhood-onset SDNS, FRNS, and SRNS were sent to 141 university, children's, and main regional hospitals in Japan in March 2010. Sixty-eight hospitals returned answers. Among them, 14 hospitals reported the actual use of RTX in 74 children by June 2010.

We analyzed the results of the questionnaires. Approval for this study protocol was obtained from the institutional review boards of the National Center for Child Health and Development. No patients who had been enrolled in a placebo-controlled double-blind clinical trial of RTX for SDNS and FRNS to gain Japanese government approval were included in this survey. Patients with two consecutive relapses of NS while receiving prednisolone on alternate days or within 15 days of its discontinuation were defined as having SDNS. Patients with two or more relapses within 6 months after initial therapy or four relapses in any 12-month period were defined as having FRNS. Patients with the inability to induce remission with 4 weeks of daily steroid therapy (60 mg/m^2 or 2 mg/kg, maximum 80 mg/day) were defined as having SRNS [16]. Relapse was defined as proteinuria 3+ by dipstick for more than 3 consecutive days. Complete remission (CR) was defined as the disappearance of proteinuria. Partial remission (PR) of SRNS was defined as more than a 50% reduction in proteinuria and recovery from hypoalbuminemia of less than 2.5 g/dl.

Statistical analysis

The Kaplan–Meier method and log-rank test were used for analyses of relapse-free survival. Statistical significance was established at $P<0.05$.

Results

Characteristics of the patients

The characteristics of the patients are summarized in Table 1. Seventy-four patients (44 male and 30 female) were administered RTX for FRNS (n=3), SDNS (n=52), and SRNS (n=19). All of the patients with SDNS/FRNS were treated with RTX under remission. The follow-up duration after the first RTX administration was 24.2±19.8 (standard deviation, SD) months (range: 8–51 months; median: 24 months). All of the patients had already been treated with various types of immunosuppressive agents. However, levamisole has not been approved in Japan and was not administered to any patients.

Overall, 59.5% of patients (n=44) had a previous (n=25) or present (n=19) history of SRNS. Of these 44 patients, 20 developed SRNS at onset (primary SRNS) and 24 had changed from steroid-sensitive nephrotic syndrome to SRNS (late SRNS). Twenty-five patients among those 44 patients changed their clinical course from SRNS to steroid-sensitive nephrotic syndrome mainly by immunosuppressive agents and/or methyl prednisolone pulse therapy. However, 19 patients had been continuously resistant to not only steroids, but also to various immunosuppressive agents, and therefore, were treated with RTX. These findings suggest that many patients with SRNS overcome steroid resistance, but still suffer from SDNS/FRNS.

The mean number of RTX administrations was 1.9±1.4 (SD) (SDNS/FRNS: 1.8±1.4; SRNS: 2.3±1.4; range: 1–7). A total of 106 courses of RTX were administered to the 74 patients, comprising 90 courses of single administration, 4 courses of 2-weekly administration, 1 course of 3-weekly administration, and 11 courses of 4-weekly administration. Of the 74 patients, 2, 3, 4, and 5 courses of RTX were given to 18, 2, 2, and 1 patient respectively. Therefore, 51 patients were treated by a single injection of RTX.

There was no standardized indication for RTX for SDNS/FRNS between the centers, but most of the patients had suffered many adverse effects of steroids and CsA, and had failed to control disease activity in spite of trials of various types of immunosuppressive agents. RTX was given during remission in all patients with SDNS and FRNS.

Table 1 Characteristics of the patients

Characteristic	Measurement
Sex (male/female) (*n*)	44/30
Age at onset, months	71±48.9
	(10–195; median: 54)
Time from onset to first RTX, months	65±43
	(2–176; median: 58)
First renal biopsy findings	
MCNS	59
FSGS	10
DMP	3
MPGN	1
Unknown	2
Relapses before RTX, number of relapses	
0–5[a]	17
6–10	26
11–20	17
>20 times	14
Previous treatment, number of patients; total number and (for SRNS)	
Cyclosporine	73 (18)
Mizoribine	47 (11)
Cyclophosphamide	32 (8)
Mycophenolate mofetil	26 (9)
Tacrolimus	8 (1)
Azathioprine	3
Methylprednisolone pulse therapy	47 (17)
Plasma exchange	12 (10)
Low-density lipoprotein apheresis	8 (3)
History of SRNS, *n*	
Previous history of SRNS	25
Present SRNS (primary/late SRNS)	19 (8/11)
Conditions at first RTX, *n*	
SDNS	52
FRNS	3
SRNS	19

Data are shown as the mean ± SD (range) or number of patients (*n*)

[a] 0 relapses includes 8 patients with SRNS

RTX rituximab, *SRNS* steroid-resistant nephrotic syndrome, *SDNS* steroid-dependent nephrotic syndrome, *FRNS* frequently relapsing nephrotic syndrome, *MCNS* minimal change nephrotic syndrome, *FSGS* focal segmental glomerular sclerosis, *DMP* diffuse mesangial proliferative glomerulonephritis, *MN* membranous nephropathy, *MPGN* membranoproliferative glomerulonephritis

Steroid and CsA-sparing effect of RTX

Steroid and CsA discontinuation after RTX administration in patients with SDNS/FRNS is shown in Fig. 1. In the SDNS/FRNS patients, 41 of the 53 patients under steroid treatment (77%) successfully discontinued steroids, after suffering various long-term adverse effects of steroid

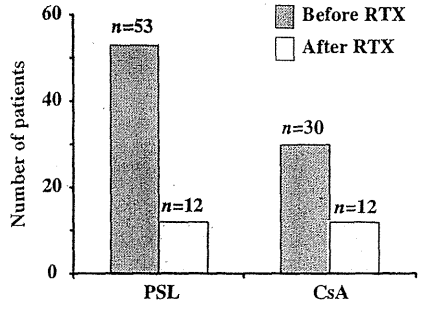

Fig. 1 Use of steroids and cyclosporine (*CsA*) in patients with steroid-dependent nephrotic syndrome/frequently relapsing nephrotic syndrome (SDNS/FRNS) after rituximab (*RTX*) administration. *PSL* prednisolone

administration. In addition, 18 of the 30 patients with SDNS/FRNS under CsA treatment (60%) discontinued CsA after RTX (Fig. 1), because they suffered from various adverse effects of CsA.

In patients with SRNS, 7 of the 17 patients under steroid treatment (41%) discontinued steroids after RTX because of CR (*n*=3), PR (*n*=2), or no efficacy (*n*=2). However, almost all of the patients with SRNS could not discontinue CsA even after RTX (Fig. 2). For 2 patients with SDNS and 2 patients with SRNS, we could not obtain information on the discontinuation of steroids because they changed hospital and were lost to follow-up.

In addition, among 37 patients (25 SDNS/FRNS and 12 SRNS) who had never discontinued steroids before RTX, 22 (16 SDNS/FRNS and 6 SRNS) had their first experience of discontinuation of steroids since onset (Fig. 3). The mean duration of steroid use was 49±36 (SD) months in these 37 patients before RTX.

Amelioration of the adverse effects of steroids and CsA

Short stature and obesity were the two main adverse effects of steroids, followed by hypertension, cataracts, and glaucoma. Hypertrichosis and CsA nephropathy were the two main adverse effects of CsA. RTX improved the various adverse effects of steroids and CsA (Fig. 4).

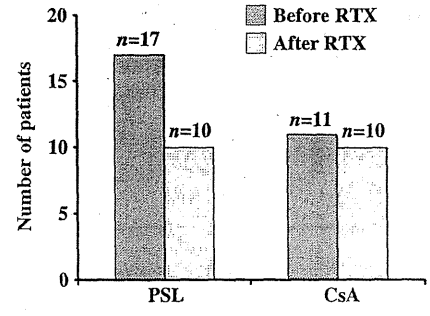

Fig. 2 Use of steroids and CsA in patients with SRNS after RTX administration. *SRNS* steroid-resistant nephrotic syndrome, *CSA* cyclosporin; *RTX* rituximab; *PSL* prednisolone

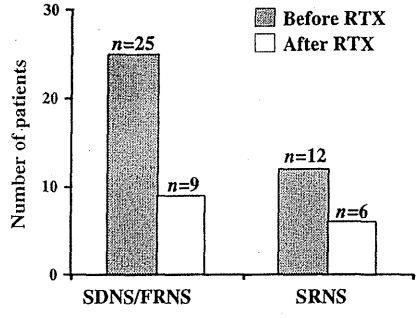

Fig. 3 Number of patients who had never discontinued steroids since disease onset. *SDNS/FRNS* steroid-dependent nephrotic syndrome/frequently relapsing nephrotic syndrome; *SRNS* steroid-resistant nephrotic syndrome

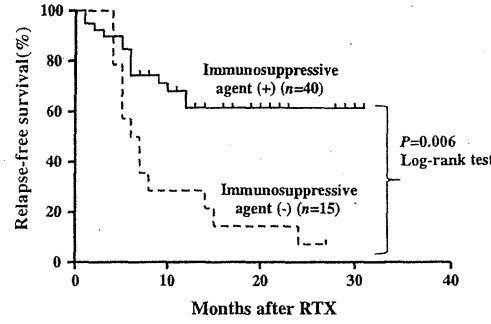

Fig. 5 Immunosuppressive agent continuation after RTX administration is beneficial for preventing relapse in SDNS/FRNS patients. *RTX* rituximab; *SDNS/FRNS* steroid-dependent nephrotic syndrome/frequently relapsing nephrotic syndrome

Relapse after RTX in SDNS/FRNS

Relapse after RTX occurred in 28 of the 55 patients with SDNS/FRNS. The time to relapse after RTX was 6.6±5.57 (SD) months (range: 1–24 months; median: 5 months). The remaining 27 patients were free from relapse for 17.3±7.8 (SD) months (range: 7–31 months; median 16 months). The mean duration of CD20 cell depletion was 5.2±1.4 (SD) months (n=48; range: 2–8 months; median: 5 months). The timing of CD20 cell recovery did not significantly differ between the relapsed (n=24) and remitted (n=16) patients (4.9±1.0 vs 5.4±1.4 months). We also examined the use of maintenance immunosuppressive agents after RTX. The patients who continued immunosuppressive agents after RTX showed a significantly lower risk of relapse (relapse occurred in 15 of the 40 patients) than the patients who discontinued immunosuppressive agents (relapse occurred in 13 of the 15 patients; Fig. 5; log-rank test, P=0.006; hazard ratio=0.201; 95% confidence interval=0.079–0.514). The mean duration of CD20 cell depletion was 5.0±1.4 (SD) months in patients who continued immunosuppressive agents (n=33) after RTX, and 4.9±1.0 (SD) months in patients without immunosuppressive agents after RTX (n=15; not significant). Immunosuppressive agents after RTX did not extend the duration of CD20-positive cell depletion. As maintenance therapy after RTX, mycophenolate mofetil, CsA, tacrolimus and mizoribine were used. However, there were no specific immunosuppressive agents that were preferable for prevention of relapse after RTX.

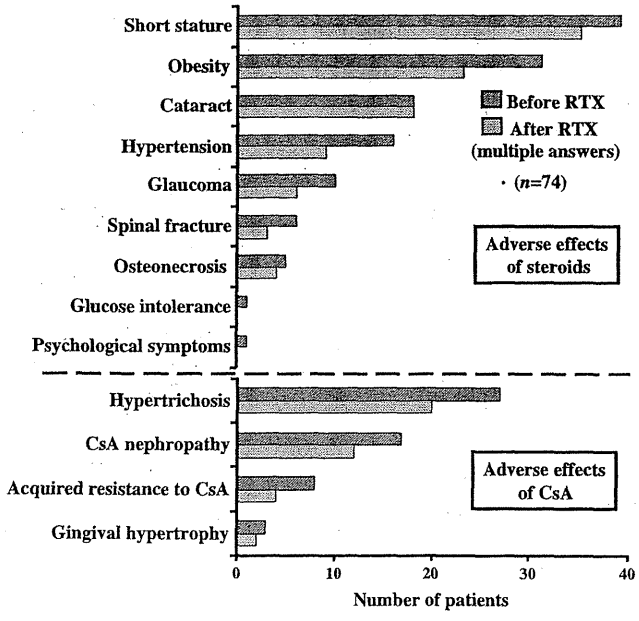

Fig. 4 Adverse effects of steroids and CsA before and after RTX administration. The diagnosis of cyclosporine nephropathy was made by biopsy. *CsA* cyclosporin; *RTX* rituximab

Effect of RTX against SRNS

All 19 patients with SRNS were resistant to various immunosuppressive agents (Table 1). None of the patients had a family history of SRNS, but 3 underwent genetic analysis, including *WT1* and *NPHS2*. After RTX therapy, CR was achieved in 6 patients (3 primary SRNS; 3 late SRNS) and PR was achieved in 6 patients (2 primary SRNS; 4 late SRNS). Conversely, 7 patients showed no response (NR) to RTX (3 primary SRNS; 4 late SRNS). One non-responder was subsequently found to have a *WT1* mutation.

Complete or partial remission were achieved 5.1±3.1 (SD) months (mean 6 months; range 1–12 months) after RTX. Seven out of 12 patients achieved CR or PR under continued CsA (additional mycophenolate mofetil in 2 and additional mizoribine in 1). The urinary protein to creatinine ratio before vs after RTX was 52±113 (SD) vs 1.01±1.3 in the CR group, 24.6±36.6 vs 7.4±9.8 in the PR group, and 73.6±160.4 vs 30.8±41.6 in the NR group.

The latest renal biopsy findings of these 19 patients were as follows: FSGS (n=11; 4 CR; 2 PR; 5 NR), minimal change nephrotic syndrome (n=8; 2 CR; 4 PR; 2 NR). Two FSGS patients had post-transplant relapse (1 CR; 1 NR). Therefore, the type of histology did not influence the response to RTX.

Table 2 Adverse events of RTX (multiple answers)

Infusion reactions		Late adverse events	
Sore throat	15	Sepsis	1[a]
Wheezing, cough	7	Granulocytopenia	2[a]
Dyspnea	7	Mild liver failure	1
Fever	4	Fever	1
Skin rash	3		
Nausea, vomiting	2		
Bradycardia	2		
Hypertension	2		
Hypotension	1		
Tachycardia	1		
Nasal stiffness	1		
Leg pain	1		

[a] Severe adverse effects: severe infection in 1 patient and granulocytopenia in 2 patients were observed, but all patients were treated adequately and recovered without sequelae

Adverse events of RTX

The adverse events of RTX are summarized in Table 2. Infusion reactions were frequently experienced, but were generally mild. Two patients developed granulocytopenia as a late severe adverse event. One of these patients had the complication of sepsis. However, both patients recovered well after treatment with granulocyte-stimulating factor and antibiotics.

Satisfaction with RTX

We also asked the parents and/or patients about their satisfaction with RTX (n=69). Overall, 29 of them (42%) felt very satisfied and 27 (39.1%) felt relatively satisfied. "Neither" and "unsatisfactory" were the responses in 15.9% and 2.9%, respectively. Overall, 81.2% had a good impression of RTX treatment.

Discussion

Rituximab treatment for nephrotic syndrome is still off-label in Japan. The results of this survey revealed that RTX has been broadly used for patients with childhood-onset refractory nephrotic syndrome. In our study, dramatic steroid- and calcineurin inhibitor-sparing effects of RTX against SDNS and FRNS are consistent with previous reports in France, India, Italy, Germany, and the United States [1–8]. Compared with these previous reports, the unique finding in our study was that immunosuppressive agents followed by a single dose of RTX could extend the relapse-free period after RTX administration.

Almost all the patients in our survey had been treated with steroid and calcineurin inhibitors for long periods, and they suffered critical adverse effects from steroid and calcineurin inhibitor administration (Fig. 4). The frequency of present or previous use of calcineurin inhibitors reached 98.6%. In addition, various types of immunosuppressive agents, methylprednisolone pulse therapy, and plasma exchange also failed to control their disease activity and allow tapering of steroid and calcineurin inhibitors. Therefore, the use of RTX was anticipated for these patients. In our survey, 41 of the 53 SDNS/FRNS patients (77%) and 5 of the 17 SRNS patients (29%) successfully discontinued steroids, which is similar to previous reports [2, 3, 6, 11]. In addition, we focused on how many patients who had never discontinued steroids since disease onset could become free from steroids after RTX administration. Remarkably, 16 the of 25 SDNS/FRNS patients and 6 of the 12 SRNS patients discontinued steroids for the first time after disease onset. This was a great benefit to the patients. Concurrently, RTX showed a considerable calcineurin inhibitor-sparing effect, because 18 of the 30 SDNS/FRNS patients were able to discontinue CsA. A reduction in the dosage of steroids and calcineurin inhibitors allowed amelioration of the serious adverse effects of both drugs (Fig. 4). Hypertension, glaucoma, and spinal fracture are urgent complications of steroid administration. Half of the patients suffering from these severe adverse events successfully recovered after RTX administration. RTX also resolved the severe adverse events of CsA, such as CsA nephropathy and hypertension, in many patients (Fig. 4). Interestingly, RTX overcame the acquired resistance to CsA. During long-term use of CsA, some patients show acquired resistance to CsA [17, 18]. Since frequent relapse under CsA increases the risk of CsA nephropathy and the dosage of steroids [19, 20], acquired resistance to CsA is problematic. In this survey, 4 of the 8 patients overcame acquired resistance after RTX administration (Fig. 4). Fujinaga et al. [12] reported the value and efficacy of maintenance therapy with CsA after a single dose of RTX, even for patients with previously CsA-refractory SDNS [10]. They also emphasized that RTX improved the drug sensitivity to CsA. CsA is one of the essential drugs used to treat SDNS/FRNS and SRNS [21, 22]. Therefore, it is a beneficial finding that RTX may recover the efficacy of CsA and allow longer use of CsA in patients with long-term intractable nephrotic syndrome. Conversely, Sinha et al. reported that two or three doses of 375 mg/m^2 of RTX is as effective as tacrolimus against intractable SDNS. Therefore, RTX could be an alternative option to calcineurin inhibitors for SDNS [23].

Although previous reports have suggested that a single dose of RTX is generally insufficient to achieve long-term remission [3, 11, 12], most of the courses in our survey involved a single administration of RTX (85%). The reason for a single dose being given may be because of the expensive price of RTX. Therefore, half of the patients with SDNS/FRNS had relapses, which occurred at 6.6±

5.5 months after RTX administration following recovery of CD20-positive cells in the peripheral blood (4.9± 1.0 months). The other half of the patients remained free from relapse for 17.3±7.8 months, even after the recovery of CD20-positive cells (5.2±1.4 months). We concluded that this difference originated from the use of immunosuppressive agents after RTX as maintenance therapy, which allowed a significant reduction in relapses after RTX (Fig. 5). However, no specific agents following RTX therapy have been proven to be beneficial for the prevention of relapses. Although use of immunosuppressive agents after RTX did not extend the duration of CD20-positive cell depletion in our study, RTX may improve drug susceptibility and recover the efficacy of immunosuppressive agents in addition to decreasing disease activity. In contrast, Kemper et al. reported that RTX allowed both steroid and maintenance immunosuppressive agents to be stopped in many patients, but in our study, RTX followed by maintenance immunosuppressive agents was significantly better for prevention of relapse [6]. This difference may be due to the ethnic background, heterogeneity of nephrotic syndrome, and the higher number of single doses of RTX in our study.

The efficacy against SRNS observed in our study is similar to the results of previous studies [1, 4, 24, 25]. In contrast, a recent randomized control trial showed that RTX did not reduce proteinuria at 3 months compared with standard therapy composed of steroids and calcineurin inhibitors [26]. The difference in the results between this randomized control trial and our study may partly be due to a longer treatment period and ethnicity. CR took 5.7±3.7 months (1–12 months, n=6) in our study.

All of the patients showed resistance to various existing therapies, except for RTX. However, CR was achieved in 6 and PR in 6 of the 19 patients. The type of histology and mode of onset (primary or late) did not influence the response to RTX. Notably, 4 patients with CR and 3 patients with PR had continued CsA after RTX administration. These findings further suggest that RTX may improve the susceptibility to CsA and decrease disease activity, which synergistically induces remission. However, RTX, especially a single injection of RTX monotherapy without immunosuppressive agents, may be insufficient to induce remission in multidrug-resistant SRNS.

Although RTX has already been used in clinical settings for over 10 years, for hematological malignancies, it can lead to rare but lethal adverse events, such as progressive multiple leukoencephalopathy, interstitial pneumonia, ulcerative colitis, and *Pneumocystis jirovecii* pneumonia [27–29]. For childhood nephrotic syndrome, there have been no reports of progressive multiple leukoencephalopathy caused by RTX, although 1 patient with SRNS died from interstitial pneumonia after RTX therapy [14]. In our survey, mild respiratory infusion reactions were the most common adverse events, as we reported previously [30]. Two patients developed granulocytopenia. In general, however, the results of this survey indicate that RTX is relatively well-tolerated.

Although RTX has considerable efficacy against childhood SDNS/FRNS and SRNS, many patients are likely to relapse, consistent with the recovery of B cells [3, 12]. Feasible solutions to solve these issues are:

1. Administration of a single dose at regular intervals
2. Consecutive multiple administrations within a short period
3. Maintenance therapy with some immunosuppressive agents after RTX

Efficacy of additional RTX administration just after the re-emergence of B cells has been reported in children with SDNS [6], but the effects of persistent B cell depletion on the developing immune system in children are unknown. Recently, Sellier-Leclerc et al. [8] reported that a 15-month CD19 depletion period induced long-term remission, even after definitive CD19 recovery in many SDNS patients, which showed a new potential for RTX. However, during the B cell deleted period, even inactivated vaccines, such as influenza virus vaccines, are impossible to use because of the lack of ability to produce antibodies [31].

Consecutive multiple administrations of RTX within a short period, such as 2 or 4 consecutive injections every week, have also been reported [3, 6, 12]. Some patients are likely to relapse after the recovery of B cells in the same manner as patients receiving a single dose of RTX [3, 6, 12]. Maintenance therapy with some immunosuppressive agents after RTX is one solution to preventing relapse after the re-emergence of B cells in the peripheral blood, consistent with previous reports [9, 10, 32]. In addition, this strategy may help to avoid repeated use of RTX and contribute to safer use and less serious adverse events of RTX.

There are some limitations to this study. It was a retrospective multi-center survey where treatment of nephrotic syndrome was heterogeneous and varied between the centers. In addition, there was no standardized indication for RTX in the treatment of SDNS/FRNS. However, most of the patients showed resistance to multiple immunosuppressive agents prior to RTX, which suggests that they suffered from intractable nephrotic syndrome.

In our study, RTX was well tolerated and reduced the burden of the severe adverse effects of steroids and CsA. In the future, more effective and harmless modes of RTX treatment are required.

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