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Ethanolamine utilization supports *Clostridium perfringens* growth in infected tissues

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Ethanolamine utilization supports *Clostridium perfringens* growth in infected tissues

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ABSTRACT

Clostridium perfringens possesses the ethanolamine (EA) utilization (*eut*) system encoded within the *eut* operon, which utilizes the EA as a carbon, nitrogen and energy source. To determine the role of the *eut* system in *C. perfringens* growth, an in-frame deletion of the *eutABC* genes was made in strain HN13 to generate the *eutABC*-deleted mutant strain HY1701. Comparison of HN13 and HY1701 growth in media supplemented with 1.0% glucose and/or 1.0% EA showed that glucose enhanced the growth of both strains, whereas EA enhanced HN13 growth, but not that of HY1701, indicating that the *eut* system is necessary for *C. perfringens* to utilize EA. The two-component regulatory system EutVW is needed to induce *eut* gene expression in response to EA whereas the global virulence regulator VirRS differentially controlled *eut* gene expression depending on glucose and EA availability. To assess the role of the *eut* system in vivo, an equal number of HN13 and HY1701 cells were injected into the right thigh muscles of mice. Mice infected with HY1701 showed fewer symptoms than those injected with HN13. The mortality rate of mice infected with HY1701 tended to be lower than for mice infected with HN13. In addition, in infected tissues from mice injected with a mixture of HN13 and HY1701, HN13 outnumbered HY1701. PCR screening demonstrated that *C. perfringens* isolated from gas gangrene and sporadic diarrhea cases carried both *eut* genes and the perfringolysin O gene (*pfoA*) as well as the phospholipase C gene (*plc*). However, *pfoA* was not detected in isolates from food poisoning patients and healthy volunteers. Culture supernatants prepared from HN13 grown in media containing 7.5% sheep red blood cells induced significantly higher *eutB* expression levels compared to those from *plc*- and/or *pfoA*-deletion mutants. Together, these results indicate that the *eut* system plays a nutritional role for *C. perfringens* during histolytic infection.

1. Introduction

Clostridium perfringens is a spore-forming anaerobic Gram-positive rod that is distributed across a wide range of environments such as soil and the mammalian gut. This anaerobe produces a variety of toxins and causes severe diseases such as gas gangrene, foodborne illness, necrotizing enteritis and septicemia. According to the productivity of α-, β-, ε-, and ι-toxins, *C. perfringens* can be grouped into five toxinotypes: A, B, C, D, and E. The majority of isolates from gas gangrene and foodborne illness belong to Type A (producing α-toxin alone) [1].

Gas gangrene is a severe disease characterized by massive tissue destruction and gas accumulation in infected tissues [2]. *C. perfringens* invades subcutaneous tissues through injured skin and produces a variety of toxins and histolytic enzymes such as collagenase and hyaluronidase, resulting in myonecrosis [3]. The necrotized tissue provides a suitable environment for *C. perfringens* growth that is anaerobic, nutrient-rich (a large amount of degraded host cells) and immunologically isolated due to poor blood supply. Although the virulence factors that are involved in tissue degradation have been analyzed in a number of studies [4], there are limited data concerning the nutritional factors that support the growth of *C. perfringens* in necrotized tissues.

Ethanolamine (EA) is a predominant component of lipid membranes in both prokaryotes and eukaryotes and is widely present in mammalian tissues and intestinal contents [5–7]. Since EA contains both carbon and nitrogen atoms, this amine can serve as carbon and/or nitrogen sources for bacteria that possess an EA utilization (*eut*) system [8–12]. The *eut* system encoded by the *eut* operon is composed of an EA

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transporter and metabolic enzymes, which form a protein complex referred to as the "carboxysome" that has structural shell proteins [13]. The carboxysome contributes to the conservation of volatile metabolites (acetaldehyde) and enzyme condensation such as ethanolamine ammonia lyase (EAL).

EA is taken into the cells by EutH and is first metabolized into acetaldehyde and ammonia by the action of EAL (EutBC) [13] before further metabolism for use as a carbon and nitrogen source, respectively. Recent studies reported that *Salmonella* and enterohemorrhagic *Escherichia coli* (EHEC) use EA in vivo, and the *eut* system is associated with their virulence [14–16]. In *Salmonella* Typhimurium, EA utilization was shown to support the growth of this enteric pathogen within the inflamed gut [17].

In *C. perfringens*, the *eut* operon is included in a strain-specific genetic island that is absent in some strains [18,19]. Notably, a genomic comparison demonstrated that two strains (strain 13 and ATCC13124) that have the potential to cause gas gangrene carried the *eut* operon, but a strain (SM101) from patients with foodborne disease did not [18]. Based on these findings, we speculated that the *eut* system could support *C. perfringens* growth in necrotic tissues since the EA could be released from cells that are lysed due to the actions of toxins and histolytic enzymes.

In this study, we aimed to determine the role of the *eut* system in *C. perfringens* growth. The results showed that genetic disruption of EAL genes reduced EA-dependent growth of *C. perfringens* both in vitro and in vivo, indicating that the *eut* system supports the growth of this pathogen in necrotic tissues.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *C. perfringens* included three genome-sequenced strains (13, ATCC13124, and SM101) as well as isolates from food poisoning and diarrhea patients and feces from a healthy individual. *C. difficile* ATCC9689, *C. tetani* KZ1113, and *C. acetobutylicum* ATCC824 were used for PCR screening of *eutB* and *pfoA*. *C. perfringens* HN13 [20], which lacks *galKT* genes, was used as a parent strain to generate *eut* gene mutants. *C. perfringens* mutants for *plc* (HN1301), *pfoA* (HN1307), *virRS* (HN1303) and six virulence-related genes (HN1314 lacking the genes for α-toxin, clostripain, collagenase, perfringolysin O and two major secretory proteins, CPE1281 and a putative cell wall lytic endopeptidase) were constructed in a previous study [20]. *Escherichia coli* DH5α was used as a host strain for plasmid construction. *E. coli* strains were cultured in lysogeny broth (LB) or on LB agar plates. *C. perfringens* strains were anaerobically grown in liquid Gifu Anaerobic Medium (GAM; Nissui Pharmaceutical Co., Tokyo, Japan), TY broth (0.8% Bacto Tryptone, 1.2% Bacto Yeast Extract, 0.5% NaCl) or on GAM plates using the AnaeroPack System (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). If necessary, chloramphenicol (Cm) was added to the media at 5 μg/ml and 10 μg/ml for *E. coli* and *C. perfringens*, respectively.

2.2. Plasmid construction and in-frame gene deletion

The *eutABC* and *eutVW* genes were deleted from the *C. perfringens* HN13 chromosome, according to counterselectable in-frame deletion methods reported by Nariya et al. [20]. In brief, fragments upstream (N-fragment) and downstream (C-fragment) of the target genes were separately amplified using the primer sets N5/N3 and C5/C3, respectively. The amplicons were mixed and fused by a second PCR (primer set N5/C3) via overlapping sequences that were incorporated into the N3 and C5 primers. The obtained product was cloned into pCM-GALK [20] that was used to construct the integration plasmids, pCM-GALK-Δ*eutABC*-IF and pCM-GALK-Δ*eutVW*-IF, respectively. These targeting plasmids were introduced into *C. perfringens* HN13 (Δ*galKT*) by electroporation. The plasmid-integrated mutants were screened by cultivation on GAM agar plates containing 10 μg/ml Cm (GAM-Cm10). Precise integration was confirmed by PCR that amplified the junctions between the integrated plasmid and the chromosome. The selected mutants were replica plated on GAM-Cm10 and GAM agar plates containing 3% galactose (GAM-GA3) and incubated anaerobically at 37 °C for 8 h. We selected Cm-sensitive colonies that grew well on GAM-GA3 as candidates for deletion mutants. These candidates were then PCR amplified using primer sets encompassing the deletion site to discriminate the expected mutants from the revertants. PCR primers used for construction of *eut* gene mutants are listed in Table S2.

2.3. In vitro growth comparison

Single colonies of *C. perfringens* HN13 (parent strain), HY1701 (Δ*eutABC*), HY1702 (Δ*eutVW*) or HN1303 (Δ*virRS*) grown on GAM agar plates were inoculated into TY broth and incubated anaerobically at 37 °C for 8 h. An aliquot (0.1 ml) of this culture was added to 10 ml freshly prepared TY broth and TY broth containing either 1% glucose (TY-G1), 1% EA (TY-EA1) or both (TY-G1/EA1). After 8 h cultivation, optical densities at 600 nm (OD_{600}) and culture pH were measured to compare the growth in the four media types. Data were collected for four independent experiments and the differences were statistically analyzed by ANOVA followed by Tukey–Kramer test. p values < 0.05 were considered to be significant. Ethanolamine hydrochloride was purchased from Sigma-Aldrich, Japan (Tokyo).

2.4. Ethanolamine ammonia lyase inhibition by hydroxocobalamin (OHCbl)

The vitamin B_{12} derivative OHCbl was reported to irreversibly inhibit EAL activity [21]. Thus, 10, 50, 100, or 200 μM OHCbl (Sigma-Aldrich Japan, Tokyo) was added to the media (TY-EA1) and the growth of *C. perfringens* HN13, HY1701, ATCC13124 and SM101 under anaerobic incubation at 37 °C was monitored across a time course. Data were collected for four independent experiments and the differences were statistically analyzed by ANOVA followed by Dunnett's test. p values < 0.05 were considered to be significant.

2.5. PCR screening of eutB and pfoA

The distributions of *eutB*, enterotoxin gene (*cpe*), *plc* and *pfoA* in 19 *C. perfringens* strains (two gas gangrene strains and 17 from patients with foodborne illness or sporadic diarrhea, as well as from a healthy subject), *C. difficile* ATCC9689, *C. tetani* KZ1113, and *C. acetobutylicum* ATCC824 were examined by PCR using the primers EutB-FW and EutB-RV for *eutB*, and pfoA-F and pfoA-R for *pfoA*. The primer sequences are listed in Table S2. Genomic DNA (20 ng) was used as a template for PCR with preheating at 98 °C for 1 min, 35 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, followed by an additional final extension at 72 °C for 3 min. PCR products were visualized by ethidium bromide staining after electrophoresis in 2% agarose gels.

2.6. Quantitative real time PCR (qPCR) for eutB

The *eutB* expression levels in HN13, HY1701, HY1702 and HN1303 cultured in TY, TY-G1, TY-EA1 or TY-G1/EA1 were compared by qPCR. To assess the functional association of the *pfoA* and *eut* system, HN13, HN1301 (Δ*plc*), HN1307 (Δ*pfoA*) or HN1314 cells were incubated in phosphate-buffered saline (PBS, pH 7.4) containing 7.5% defibrinated sheep red blood cells (RBCs) for 2 h at 37 °C. After incubation, the mixtures were centrifuged at 10,000 g for 5 min at 4 °C and then filtered. Each culture supernatant was equally mixed with fresh TY medium. HN13 was cultured in the media for 2 h at 37 °C. Total RNA was extracted by the hot phenol method [22]. RNAs were further

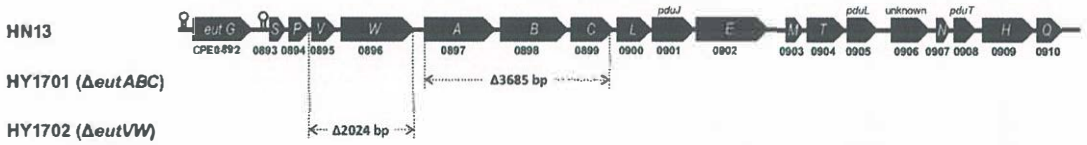

Fig. 1. Diagram of the *eut* operon in *C. perfringens* HN13. The genetic organization of the *eut* operon in HN13 is identical to that of *C. perfringens* strain 13. The genes encoding products related to ethanolamine catabolism are labeled alphabetically. Deletion sites in HY1701 and HY1702 are shown below the gene map. The previously reported anti-terminator elements containing the ANTR motif (AGCAANGRRGCUY) are shown by a hairpin structure. The coding function of each gene is listed in Table S3.

purified using an RNeasy CleanUp Kit (Qiagen) and treated with TURBO DNA-*free* (Ambion) to remove contaminating DNAs. Total RNA was reverse-transcribed using a PrimeScript RT reagent kit (Takara Shuzo Co., Ltd.) with random hexamers at 37 °C for 15 min. Reverse transcription was terminated by heating the mixtures at 85 °C for 5 s. The cDNA products were subsequently amplified using SYBR Premix Ex-*Taq*II (Takara) under the following conditions: preheating at 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s in a StepOnePlus apparatus (Applied Biosystems). All samples were run in triplicate. Threshold cycle values were normalized to the levels of 16S ribosomal RNA gene transcripts, and changes in gene expression were calculated using the $2_T^{-\Delta\Delta C}$ method [23]. Data were collected for four independent experiments and the differences were statistically analyzed by ANOVA followed by Tukey–Kramer test. p values < 0.05 were considered to be significant. The oligonucleotide sequences of the primers used for *eutB* qPCR are listed in Table S2.

2.7. Comparison of C. perfringens growth in a gas gangrene mouse model

C. perfringens HN13 and HY1701 were cultured anaerobically in 10 ml TY-G1 broth at 37 °C for 12 h. After the cultures were centrifuged at 5000 g for 5 min at room temperature, the pellets were washed once with PBS, and finally resuspended in PBS with the cell density adjusted to 1×10^9 cfu/ml. The pathogenic potential to cause gas gangrene was compared between the strains according to the gas gangrene mouse model using *C. septicum* reported by Kennedy et al. [24]. Five 8-week-old male ddY mice per group were used in this study. The mice were deeply anesthetized and intramuscularly injected with 100 µl (1×10^8 cells) cell suspension of HN13 or HY1701 into the right thigh. The mice were observed every hour to evaluate malaise, limping, swelling and blackening of the right thigh and foot pad as parameters for the severity of infection and scored using the following grades: slight, 0; moderate, +1; and severe, +2. Cumulative scores of five mice were compared between the HN13- and HY1701-injected groups. To compare the competitive growth, 5.0×10^7 cells of both *C. perfringens* HN13 and HY1701 were mixed and injected into the right thighs of five mice. At 24 h after infection, the right thigh muscles were aseptically collected and homogenized in 10 vol of PBS. Serial dilutions of the homogenates or inocula of *C. perfringens* were made with PBS and 100µl each of the dilution was plated on GAM agar plates. The plates were then anaerobically incubated at 37 °C overnight, and the viable cell number per gram of infected muscle was calculated from the colony counts. Multiplex colony PCR using the primer sets EutB-FW/EutB-RV and eutABC-SQ1/eutABC-SQ2, which amplify *eutB* and confirm *eutABC* deletion, respectively, was performed on 96 colonies to discriminate parent HN13 from HY1701 cells. Differences in the HN13/HY1701 ratio of the inoculum and the infected muscles were statistically examined by chi-square test. The cumulative pathological scores were statistically compared by log-rank test. p values < 0.05 were considered to be significant.

2.8. Ethics

All animal experiments were performed according to Kagawa University guidelines for animal experiments. The experimental protocol was approved by the animal experiment committee of Kagawa University (approved number: 156–1).

3. Results

3.1. Genetic deletion of eutABC and eutVW in C. perfringens HN13

The genetic organization of the *eut* operon in *C. perfringens* HN13 is shown in Fig. 1. This locus contains 15 *eut* genes, 3 genes for 1,2-propandiol utilization and a gene of unknown function (Table S3). The *eutB* and *eutC* encode EAL, which is a key enzyme for EA utilization. The *eutV* and *eutW* encode a response regulator and a sensor histidine kinase, respectively, which constitute a two-component regulatory system (TCS). We made an in-frame deletion of a 3685 bp fragment containing *eutABC* to eliminate *eut* function to generate strain HY1701. In strain HY1702, a 2024 bp fragment containing *eutVW* was deleted in-frame to assess the function of this TCS in EA sensing.

3.2. Role of the eut operon for in vitro growth of C. perfringens

The growth of HN13, HY1701, HY1702 and HN1303 was assessed in TY broth alone and TY broth containing glucose (TY-G1), ethanolamine (TY-EA1) or both (TY-G1/EA1). HN13 growth was significantly enhanced in TY-G1, TY-EA1 and TY-G1/EA1 compared to TY broth ($p < 0.05$; Fig. 2A) and the culture pH decreased concurrently with growth (Table S4). Meanwhile, compared to TY broth, HY1701 and HY1702 growth was enhanced in TY-G1 and TY-G1/EA1 but not TY-EA1. These results indicate that C. *perfringens* can use EA as an energy source and that the *eut* system is essential for utilization of EA. Glucose (1%) enhanced the growth of all test strains much more than did EA (Fig. 2B). This growth promotion by glucose was reduced in *eut*-positive strains (HN13 and HN1303) when EA was supplied (Fig. 2A). In addition, *virRS* deletion reduced the growth-promoting effect of glucose (Fig. 2B).

3.3. Inhibitory effect of OHCbl on C. perfringens EA utilization

EAL is a key enzyme in the *eut* system and requires adenosylcobalamin as a cofactor. The irreversible EAL inhibitor hydroxocobalamin (OHCbl) was added to TY-EA1 broth at a final concentration of 10–200µM and growth of *C. perfringens* HN13, HY1701, ATCC13124 and SM101 was monitored by OD_{600} of 8 h cultures. OHCbl dose-dependently inhibited the growth of *eut*-positive *C. perfringens* (HN13 and ATCC13124) in TY-EA1 (Fig. 3). In contrast, OHCbl had no effect on the growth of the *eut*-negative *C. perfringens* strains HY1701 and SM101.

3.4. Distribution of the eut operon in clostridia

PCR screening of *eutB* was performed to examine the distribution of the *eut* system in *C. perfringens* and related species. Multiplex PCR detected *eutB* in gas gangrene (13 and ATCC13124) and plasmid-*cpe* positive strains, whereas no product was generated from chromosomal *cpe*-positive strains (Table 1). The *plc* gene was amplified from all *C. perfringens* strains tested, whereas *pfoA* encoding the pore-forming perfringolysin O (PFO) was present in *C. perfringens* from gas gangrene,

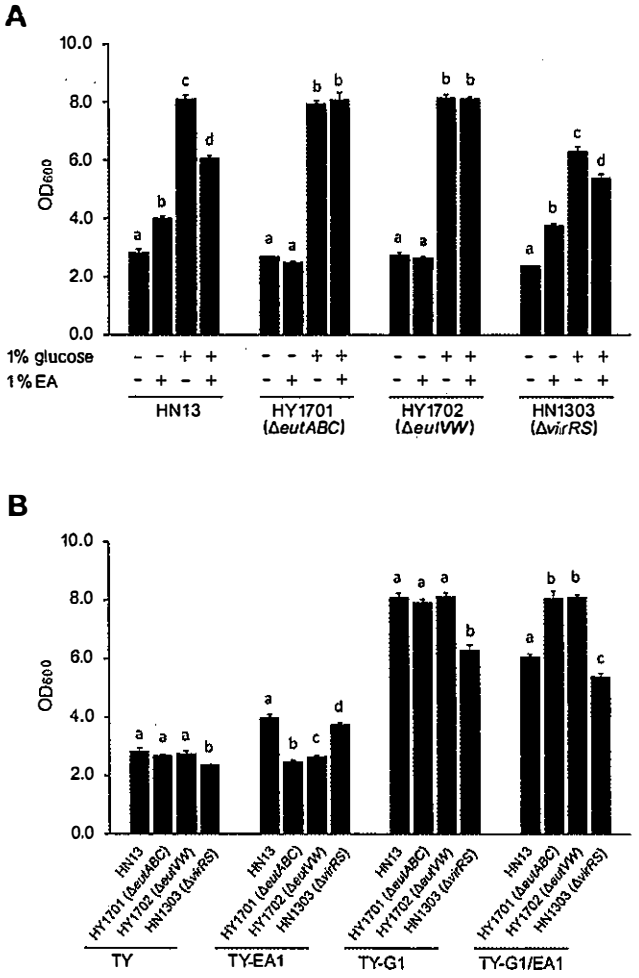

Fig. 2. *C. perfringens* growth enhancement by EA. (A) Growth of each test strain in different media types. The presence and absence of glucose or EA in the culture is indicated by + and -, respectively. (B) Growth comparisons among the test strains in each media type. Growth of 8 h cultures in TY, Tryptone Yeast broth; TY-G1, TY containing 1% glucose; TY-EA1, TY containing 1% EA or TY-G1/EA1, TY containing 1% glucose and EA was measured at OD_{600}. The data are expressed as mean ± standard deviations from four independent replicates. The samples labeled with different letters are significantly different from each other ($p < 0.05$).

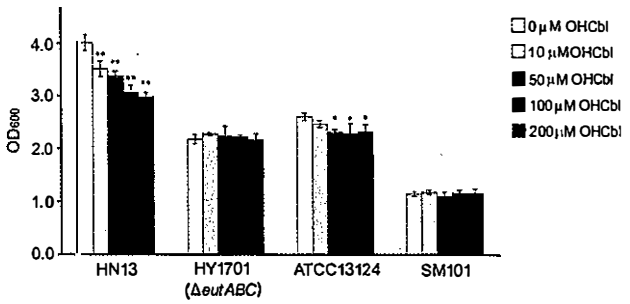

Fig. 3. Inhibitory effect of OHCbl on *C. perfringens* growth *C. perfringens* strains were cultured in TY containing 1% EA and indicated concentration of OHCbl. The data are expressed as mean ± standard deviations from four independent replicates. Significant differences relative to cultures without OHCbl for each strain are indicated by ** ($p < 0.01$) and * ($p < .0.05$).

Table 1
PCR survey for *eutB*, *cpe* and *pfoA* in clostridia.

Strain	Source	*eutB*	*plc*	*cpe*	*cpe location*	*pfoA*
Clostridium perfringens						
Strain 13	Soil (known to cause gas gangrene in mice)	+	+	–		+
ATCC13124	Type strain, gas gangrene	+	+	–		+
SM101	Type strain, NCTC8798 derivative	–	+	+	C	–
NCTC8798	Food poisoning	–	+	+	C	–
NCTC8239	Food poisoning	–	+	+	C	–
W4232	Food poisoning	–	+	+	C	–
W5837	Food poisoning	–	+	+	C	–
W6235	Food poisoning	–	+	+	C	–
OSAKA1	Food poisoning	–	+	+	C	–
OSAKA2	Food poisoning	–	+	+	C	–
OSAKA3	Food poisoning	–	+	+	C	–
OSAKA4	Food poisoning	–	+	+	C	–
CP093	Food poisoning	–	+	+	C	–
CP094	Food poisoning	–	+	+	C	–
CP095	Food poisoning	–	+	+	C	–
F4969	Sporadic diarrhea	+	+	+	P	+
FS603	Sporadic diarrhea	+	+	+	P	+
T1	Food poisoning	+	+	+	P	–
MR2-4	Healthy	+	+	+	P	–
Other clostridia						
C. difficile ATCC9689		+	–	–		–
C. tetani KZ1113		+	–	–		–
C. acetobutylicum ATCC824		+	–	–		–

C: Chromosome, P: Plasmid.

soil and sporadic diarrhea cases, but not food poisoning-derived strains. Other tested clostridia, including *C. difficile*, *C. tetani*, and *C. acetobutylicum* possessed the *eut* operon but *C. botulinum*, *C. novyi*, and *C. cellulolyticum* were negative for *eutB*.

3.5. Regulation of the eut operon

Expression of *eutB* was monitored by qPCR to elucidate the mechanism of *eut* operon regulation in *C. perfringens*. Glucose decreased *eutB* expression in HN13 (Fig. 4A). In the presence of EA, *eutB* expression was increased in HN13 cells independent of glucose. EA-dependent *eutB* induction was abrogated in HY1702, in which the *eut* operon TCS genes (*eutVW*) were deleted (Fig. 4A). Expression of *eutB* was also induced by EA in HN1303 (Δ*virRS*) to a similar level as that of HN13. These results indicated that TCS encoded by *eutVW* induces expression of the *eut* operon in response to extracellular EA independently of VirRS. The *eutB* expression by HN1303 cultured in TY was significantly lower than that by HN13 (Fig. 4B). Although glucose repressed *eutB* expression in HN13, *virRS* deletion cancelled this inhibition. Therefore, we concluded that VirRS represses *eut* gene expression under glucose-rich conditions, but EA canceled this negative regulation. However, VirRS appeared to induce *eut* operon expression when both glucose and EA were unavailable.

3.6. Role of PLC and PFO in the activation of eut operon

Of the six *eut*-positive *C. perfringens* strains, four also possessed *pfoA*. To determine the role of PLC and PFO in the activation of *eut* oepron, 7.5% sheep RBC in PBS was incubated with HN13, HN1301 (Δ*plc*), HN1307 (Δ*pfoA*) or HN1314 (Δ6 virulent genes including *pfoA* and *plc*). After centrifugation of the mixtures, the supernatants were added 1:1 into TY with *C. perfringens* HN13. The *eutB* expression in these cultures was then compared by qPCR. The *eutB* expression level in HN13 after 2 h and 4 h incubation with HN1301-treatd RBCs was lowest among the

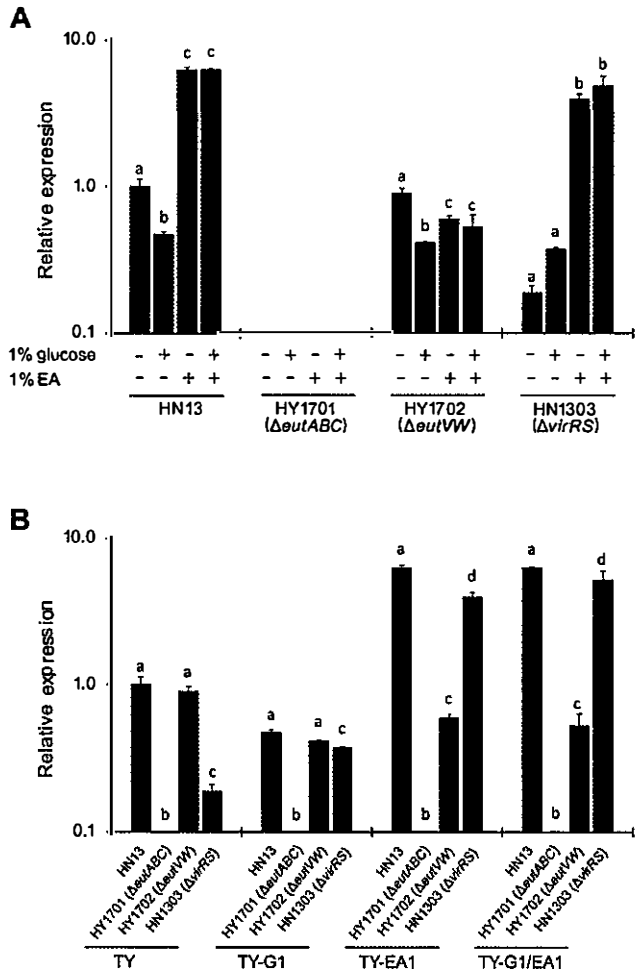

Fig. 4. Quantitative real-time PCR to monitor *eutB* expression in *C. perfringens*. (A) The *eutB* expression level in each test strain in different media types. The presence and absence of glucose or EA in the culture is indicated by + and -, respectively. Expression levels were normalized with respect to 16S rRNA. (B) Comparison of *eutB* expression levels among the test strains in each media type. The data are expressed as mean ± standard deviations from three independent replicates. The samples labeled with different letters are significantly different from each other ($p < 0.05$).

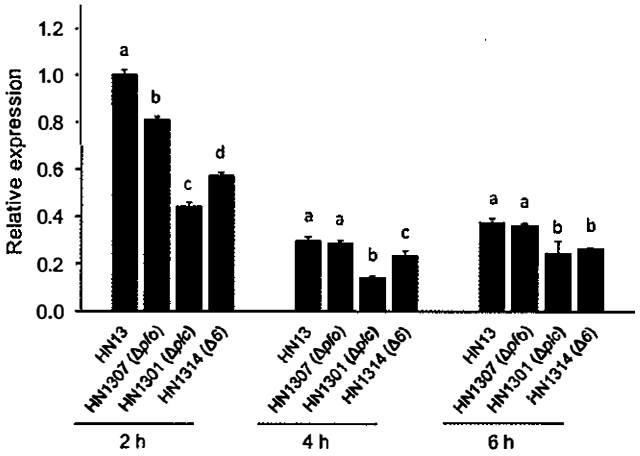

Fig. 5. Comparison of *eutB* expression in *C. perfringens* HN13 incubated with HN13-, HN1307-, HN1301- or HN1314-treated sheep RBCs. Relative *eutB* expression levels were compared among the samples at each indicated time point. Expression levels were normalized with respect to 16S rRNA. The data are expressed as mean ± standard deviations from four independent replicates. The samples labeled with different letters are significantly different from each other ($p < 0.05$).

samples. In addition, the *eutB* expression level in HN13 after a 2 h incubation with HN1307-treated RBCs was significantly lower than that in cells treated with HN13-treated RBCs (Fig. 5). These results indicate that PLC has a major role in the release of inducers for *eut* operon from RBCs and that PFO facilitates its action. Although HN1314 is negative for both *plc* and *pfoA*, the *eutB* expression level in HN13 after 2 h and 4 h incubation with HN1314-treatd RBCs was significantly higher than that with HN1301-treatd RBCs. This result might reflect the low availability of both glucose and EA in HN1314-treated RBC samples, thus inducing the positive effect of VirRS on *eutB* expression as described above.

3.7. Role of the eut operon in C. perfringens growth in a gas gangrene mouse model

The in vivo growth of *C. perfringens* HN13 and HY1701 was compared in a gas gangrene mouse model. An equal number of each cell type (1×10^8 cells) was injected intramuscularly into the right thighs of male 8-week-old ddY mice (five each for HN13 and HY1701). The cumulative pathological scores for malaise, limping, foot pad swelling and blackening of the right thigh and foot pad were significantly lower in mice infected with HY1701 compared to those infected with HN13 (Fig. 6). Although the survival rate was not significantly different between the groups, the mortality rate of the mice infected with HY1701 tended to be lower than that of the HN13-infected mice (0% vs. 40%).

Finally, the in vivo growth of HN13 and HY1701 was compared in a competitive fashion. The right thighs of the mice were intramuscularly injected with 0.1 ml of a cocktail containing 5.0×10^7 cells each of HN13 and HY1701. After 24 h, the number of viable HN13 and HY1701 cells in the infected muscles was compared by colony PCR. The total number of *C. perfringens* cells recovered from infection sites was $9.5 \pm 5.9 \times 10^5$ cfu/g of tissue. No significant difference was found in the ratio of HY1701 and HN13 in the inoculum (54.3% vs 45.7%; Fig. 7). However, the ratio of HY1701 cells in the infected thigh muscles was significantly lower than that of HN13 ($p < 0.001$). The percentage of HY1701 and HN13 in infected tissues was 30.0 ± 6.9% and 70.0 ± 7.0%, respectively.

4. Discussion

C. perfringens causes gas gangrene, and its histolytic toxins such as α-toxin (PLC) and θ-toxin (PFO) play a critical role in disease establishment and progression [1,25,26]. However, there are limited data concerning the nutritional factors that support *C. perfringens* growth in necrotic tissues. In this study, we used a mouse gas gangrene model to show that EA utilization plays an important role in *C. perfringens* growth in vivo.

The in vitro growth comparison demonstrated that EA promoted growth of HN13, but not of HY1701 in which the *eut* operon was deleted, indicating that the *eut* operon is the sole system for EA use by *C. perfringens*. Accordingly, *eut*-negative SM101 also did not utilize EA. Plasmid complementation of HY1701 with *eutABC* did not restore EA utilization (data not shown), indicating that EAL supplied *in trans* is unlikely to be incorporated into the carboxysome. Interestingly, EA growth promotion differed even among *eut*-positive *C. perfringens* as seen by weaker EA-dependent growth enhancement of ATCC13124 compared to HN13 (Fig. 3). This result indicates that under conditions of limited glucose the ATCC13124 strain may preferentially use nutrients other than EA. Correspondingly, the inhibitory effect of OHCbl on EA utilization by ATCC13124 was weaker than that for HN13.

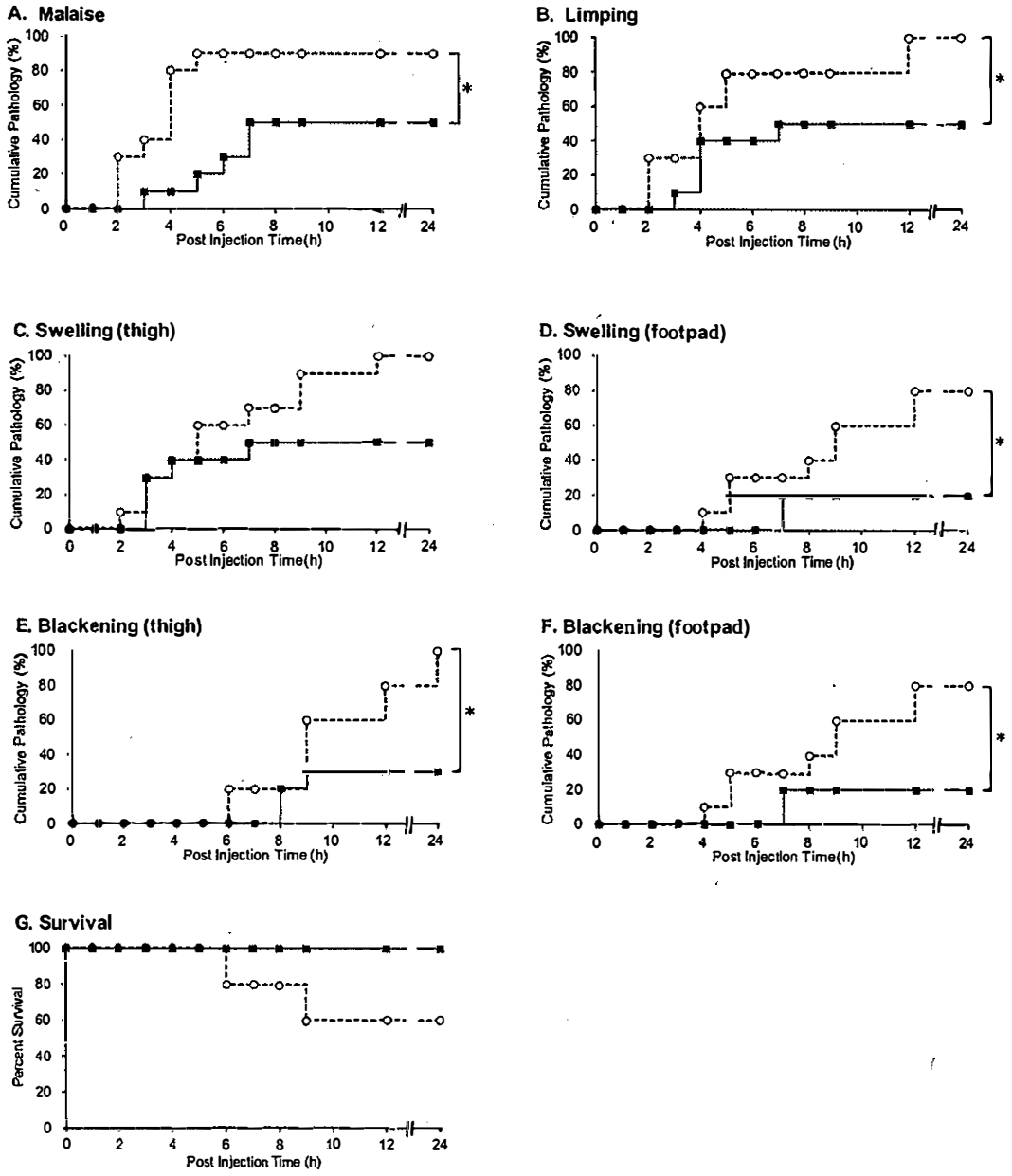

Fig. 6. Comparison of pathogenic potential of *C. perfringens* HN13 and HY1701 (Δ*eutABC*) in a mouse gas gangrene model. Cumulative pathologic scores and survival rates were recorded up to 24 h after inoculation with *C. perfringens* strains HN13 (open circles) or HY1701 (filled squares). Five mice were used for each group. *Significantly different between the groups ($p < 0.05$).

As shown in our gas gangrene mouse model, the *eut* system supported C. *perfringens* growth in necrotic tissues. The symptoms were more severe and mortality was higher in mice infected with HN13 compared to those infected with HY1701 (Δ*eutABC*). However, *plc* and *pfoA* expression levels in EA-supplemented media were similar between HN13 and HY1701 (data not shown). Since EA levels are expected to be high in necrotic tissues, the severe symptoms and higher mortality rate for HN13-infected mice might be partly attributable to the energy supply from EA afforded by the *eut* system, which maintains *C. perfringens* growth in vivo.

OHCbl is an irreversible inhibitor of adenosylcobalamin-dependent EAL [21]. Here we showed that OHCbl could dose-dependently abrogate EA growth enhancement seen for the *eut*-positive *C. perfringens* strains HN13 and ATC13124. OHCbl is used as a first line drug for cyanide poisoning [27], but could also be a supportive drug for treating myonecrosis induced by *eut*-positive *C. perfringens*. However, intraperitoneal injection of OHCbl did not improve symptoms in our mouse gas gangrene model (data not shown). Blood supply to myonecrotic tissues in C. *perfringens* infections is known to be poor due to the massive platelet aggregation that occurs in blood vessels [28]. Thus, development of a method to promote efficient delivery of OHCbl to necrotic tissue is likely needed to evaluate the therapeutic effect of this drug for *C. perfringens* myonecrosis. *C. perfringens* also causes necrotic enteritis. As such, oral administration of OHCbl might be an alternative treatment for inhibiting *C. perfringens* growth in the gut.

Expression of the *eut* operon in *Enterococcus faecalis* and *Listeria monocytogenes* are uniquely controlled by a post-transcriptional antitermination mechanism involving TCS and/or riboswitch [11,29,30].

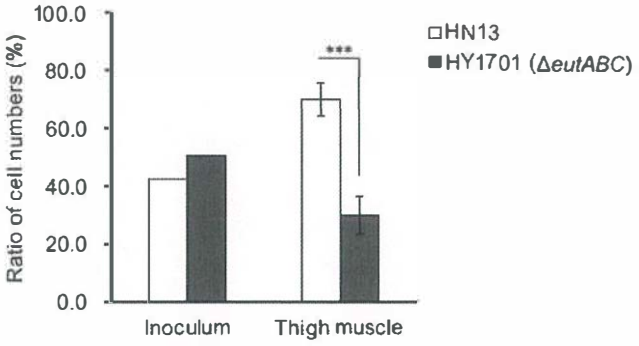

Fig. 7. Abundance of *C. perfringens* HN13 and HY1701 (Δ*eutABC*) in infected muscles. The ratios of the cell number of *C. perfringens* HN13 (open columns) and HY1701 (filled columns) were compared in the inoculum used for the mouse gas gangrene model and in infected right thigh muscles. Five mice for each sample were examined. ***Significantly different between the samples ($p < 0.001$).

EutV is predicted to bind to the AmiR and NasR transcriptional antiterminator regulator (ANTR) motif sequence [31], located in intergenic regions upstream of *eutG* and *eutS*, to eliminate terminator structures (Fig. 1). The expression of the *eut* operon is also controlled by global virulence regulators such as Csr in *Salmonella* Typhimurium [32] and Fsr in *Enterococcus faecalis* [33]. The VirRS system in *C. perfringens* is recognized as a global virulence regulator that controls expression of *pfoA*, VR-RNA, *plc* and many other virulent-related genes [22,34,35]. Yamaguchi et al. reported that VirRS negatively regulated the *eut* operon in *C. perfringens* strain 13 [36]. We also consistently observed a negative effect of VirRS on *eut* operon expression under glucose-rich conditions (Fig. 4). However, this suppressive effect by VirRS was canceled when EA was available. The *eut* gene induction by EA was dependent on TCS composed of EutVW. Interestingly, VirRS up-regulated *eutB* expression in the absence of glucose and EA. This result might reflect an adaptation to poor nutritional conditions, and that maintenance of basal expression of the *eut* operon enables *C. perfringens* to rapidly respond to the presence of EA. These results indicate that histolytic *C. perfringens* preferentially utilizes EA and regulates the expression of the *eut* operon by EutVW and VirRS depending on glucose and EA availability.

Phosphatidylethanolamine (PE) is a major membrane component of eukaryotes and prokaryotes [5–7]. PLC degrades PE to diacylglycerol and phosphoethanolamine (pEA). The released pEA might be further metabolized to EA by phosphatase derived from lysed host-cell or *C. perfringens* itself. It is also possible that pEA induces signal transduction via EutVW. As shown in Fig. 5, PLC has a major role in the release of inducers for *eut* operon from sheep RBCs. The significantly lower level of *eutB* induction by HN1307 (Δ*pfoA*)-treated sheep RBCs compared to HN13-treated sheep RBCs indicated that PFO facilitates PLC-mediated PE degradation. Taguchi et al. reported that *C. perfringens* PLC did not hydrolyze PE unless other lipids such as phosphatidylcholine and other membrane components were present [37]. Therefore, PFO is predicted to promote PLC-mediated degradation of PE by releasing membrane components from host cells. Under *plc*-negative and *pfoA*-positive conditions, EA may not be available, but other nutrients such as glucose could be released from RBCs by the pore-forming action of PFO. Nutrients other than EA released from lysed RBCs might elicit *eutB* suppression by VirRS (Fig. 2). We showed that *eutB* expression in HN13 incubated with HN1314 (Δ6)-treated RBCs was higher than that with HN1301 (Δ*plc*)-treated RBCs (Fig. 5). Simultaneous deletion of *plc* and *pfoA* (as in HN1314) is predicted to decrease the availability of both glucose and EA from RBCs, eliciting a positive effect of VirRS on *eutB* expression as observed in TY without glucose and EA as shown in Fig. 2.

Although PLC is known to play a critical role in *C. perfringens* gas gangrene, the pathogenic role of PFO is controversial [38–40]. However, PLC is thought to facilitate the pore-forming action of PFO [26], which in turn promotes PE hydrolysis by PLC. Therefore, the *eut* system is predicted to be involved in the establishment and progression of myonecrotic lesions by *pfoA*-positive *C. perfringens*. Canard et al. reported that in the *C. perfringens* chromosome the *pfoA* region is a highly variable [38]. Thus, comparisons of the pathogenic potential of *eut*-positive *C. perfringens* that have varying PFO productivity would be valuable.

In conclusion, in this study we showed that the *C. perfringens eut* system supports growth of this anaerobe in vitro and in vivo. Deletion of the *eut* system attenuated competitive growth in infected muscles and disease severity. Histolytic *C. perfringens* appears to preferentially utilize EA. This study indicated that the global virulence regulator VirRS is involved in *eut* operon expression in a complex manner that depends on glucose and EA availability. These findings indicate that the *eut* system might be a therapeutic target for gas gangrene and necrotizing enteritis caused by *C. perfringens*, and that the EAL inhibitor OHCbl could be a candidate drug.

Conflicts of interest

None.

Acknowledgements

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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.micpath.2018.04.017.

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