Organization and transcriptional regulation of the ethanolamine utilization operon in Clostridium perfringens

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ABSTRACT

Clostridium perfringens is a Gram-positive anaerobic rod and is the chief causative agent of gas gangrene. The development of gas gangrene requires rapid growth of *C. perfringens* and the expression and production of numerous enzymes/toxins that degrade host tissues. The host cell membranes are considered to provide a rich source of ethanolamine (EA), which can be utilized as an energy source under hostile conditions by several bacterial pathogens. This study revealed the following: [1)] *C. perfringens* can utilize EA as an energy source for growth; a two-component system located in the EA utilization (*eut*) region regulates the expression of the *eut* operon; [2)] the VirR/VirS system and the *virX* system also affect the transcription of the *eut* operon, which suggests that the *eut* operon of *C. perfringens* plays a role in its pathogenicity by managing energy production from EA.

Key words *Clostridium perfringens*, ethanolamine utilization, gene regulation

INTRODUCTION

Clostridium perfringens is a Gram-positive spore-forming anaerobic bacterium that is commonly found in the gastrointestinal tracts of humans and animals, as well as in soil and sewage. *C. perfringens* has been shown to cause human diseases, such as gas gangrene, food poisoning, necrotizing enterocolitis in infants, and enteritis necroticans, due to the production of various toxins [1)2)].

Gas gangrene is a life-threatening disease in humans. The pathogenesis of the disease involves the entry of *C. perfringens* into the body cavity via a major traumatic injury or surgical wound [3)]. Massive tissue damage resulting from the injury facilitates establishment of the anaerobic conditions that are required for the growth of *C. perfringens* [2)3)]. Rapid growth of *C. perfringens* in the human body is accompanied by the production of numerous extracellular toxins and enzymes, which leads to gas production, extensive necrosis, and severe tissue damage.

Phosphatidylethanolamine is an abundant phospholipid in both mammalian and bacterial cell membranes and can be readily broken down into glycerol and ethanolamine (EA) by phosphodiesterases [4)]. The cell membranes of the skeletal muscle and/or connective tissue cells can provide rich sources of EA. Under hostile conditions, several bacterial species, including *Enterococcus faecalis*, *Escherichia coli*, and *Salmonella* species, can use EA as a sole source of carbon and/or nitrogen [5-7)]. Therefore we hypothesized that *C. perfringens* could use EA as an energy source for growth, leading to the development of gas gangrene in the human body.

Korbel *et al*. reported that three of the most hazardous food-borne pathogens, *Listeria monocytogenes*, *C. perfringens*, and *Salmonella enterica*, carry highly similar EA utilization operons (*eut* operons) in their genomes, whereas the *eut* genes

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Abbreviations: ANTAR, AmiR and NasR transcription antitermination regulators; EA, ethanolamine; eut, ethanolamine utilization; HK, histidine kinase; TCS, two-component system; RR, response regulator.

are absent in most other prokaryotes [8]. Based on these findings, it was predicted that the eut pathway is an important genomic determinant for pathogenicity associated with food poisoning. Bacteria have to sense and respond adequately in order to survive fluctuating environmental condition. Many bacteria predominantly use two-component systems (TCSs) for signaling. A TCS consists of a membrane-integrated histidine kinase (HK), which perceives a stimulus, and cytoplasmic response regulator (RR), which mediates the output, generally an alteration in gene expression. HK and RR exchange information via conserved phosphorylation and dephosphorylation reactions [9)10)]. The genome of *C. perfringens* contains 28 predicted TCS systems. VirS/VirR(CPE1500/CPE1501) is one of these TCSs. Many genes for virulence factors in *C. perfringens* are regulated by the two-component VirR/VirS-VR-RNA system and the *virX* system [11-14)]. VirR/VirS directly at least five genes, including *proA* (encoding theta-toxin), *vrr* (RNA regulator VR-RNA), *ccp* (encoding alpha-clostripain), *virT* (RNA regulator) and *virU* (RNA regulator) [11)13)-15)16)]. Furthermore, A recent microarray analysis suggested that 147 genes, including genes (*plc*: phospholipase C, *cola*; collagenase, *nanJ*; sialidase,) *nanI*; sialidase, *nagL*; hyaluronidase) for virulence factors, enzymes, transporters and energy production, were regulated by VR-RNA molecule [15)]. Previous studies have reported that the VirR/VirS-VR-RNA system affects the expression of the genes for nutrient metabolism (e.g., *myo*-inositol utilization, hemeoxygenase, acid phosphatase, citrate utilization) [17)-20)]. These studies strongly suggested that the VirR/VirS-VR-RNA system controls multiple cellular functions that are necessary in the development of severe histolytic disease in humans. The *virX* gene encodes 51 amino acids that form a peptide, and it positively regulates the *pfoA*, *plc* (encoding alpha-toxin), and *colA* (encording kappa-toxin). It was shown that *virX* acts as a small RNA regulator for the control of virulence in C.perfringens [12)21)].

In this study, we characterized the genes encoding eut function in *C. perfringens*. We revealed the presence of the TCS in this region and demonstrated the regulatory effect of the TCS_{eut} on the *eut* operon. We also examined the effect of the VirR/VirS-VR-RNA system and the *virX* system on the expression of the *eut* operon, which suggested the association of EA metabolism with the pathogenicity of *C. perfringens*.

MATERIALS AND METHODS

Strains, media and culture conditions

All *C. perfringens* strains used in this study (Table 1) were cultured in GAM (Gifu Anaerobic Medium; Nissui, Japan) and YT medium or minimal medium at 37℃ under anaerobic conditions. The minimal medium was modified from the original [25)] and contained (per liter) 0.5 g DL-α-alanine, 5.0 g L-arginine, 0.5 g L-aspartic acid, 0.4 g L-cystine, 0.5 g glycine, 1.0 g L-glutamic acid, 0.25 g L-histidine, 0.2 g L-hydroxyproline 0.5 g DL-isoleucine, 0.5 g L-lysine, 0.5 g L-leucine, 0.35 g DL-methionine, 0.25 g L-proline, 0.5 g DL-phenylalanine, 0.75 g

Table 1. Strains and plasmids

Strains/plasmid	Characteristics	Source/reference
C. perfringens		
13	Wild-type strain (type A)	22)
TS133	Strain 13 *virR*::Tet^r	13)
TS140	Strain 13 Δ*vrr* Em^r	14)
TS186	Strain 13 ::*virX* Em^r	12)
TCS11	Strain 13 ΔCPE0895-0896 Em^r	This study
0897mutant	Strain 13 *eutA*::Em^r	This study
E.coli		
DH5α	*supE*44 Δ*lacU169*(φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Takara Bio Inc.
Plasmids		
pJIR418	E. coli-C. perfringens shuttle vector, Cm^r Em^r	23)
pBT405	pJIR418 Ω(*Pst*I 4.3-kb strain 13 genomic library) (virR+ virS+ complementation vector), Cmr	Shimizu, unpublished
pSB1031	pJIR418 Ω(PCR-amplified 637-bp fragment) (*vrr*$^+$ complementation vector)	24)
pTS907	pJIR418 Ω(*Hin*dIII 1.4-kb strain 13 genomic library) (*virX*$^+$ complementation vector)	12)
pTCS11	pJIR418 Ω(*Hin*dIII 4.6-kb strain 13 genomic library) (*TCSeut*$^+$ complementation vector)	This study
pUC18/19	Cloning vector, Amp^r *lacZ*' pMB 1 ori	Takara Bio Inc.

[#]Tet^r, resistance to tetracycline; Em^r, resistance to erythromycin; Cm^r, resistance to chloramphenicol; Amp^r, resistance to ampicillin.

DL-serine, 0.5 g DL-threonine, 0.25 g L-tryptophan, 0.25 g L-tyrosine, 0.375 g DL-valine, 0.01 g uracil, 0.0174 g adenine sulfate, 0.25 g ascorbic acid, 0.0005 g vitamin B2, 0.001 g D-pantothenic acid, 0.0005 g pyridoxamine, 0.000005 g D-biotine, 0.001 g nicotinic acid, 0.001 g thiamine hydrochloride, 2.85 g Na_2HPO_4, 0.7 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnCl_2 \cdot 4H_2O$, 0.05 g $ZnSO_4 \cdot 7H_2O$, and 0.05 g $FeSO_4 \cdot 7H_2O$. *E. coli* DH5α was cultured using the standard procedure. Plasmid pUC18/19 and pJIR418 [23] were used for general gene cloning in *E. coli* and transformation of *C. perfringens*, respectively. Erythromycin (50μg/ml), chloramphenicol (25μg/ml), and ampicillin (50μg/ml) were added to the medium, as appropriate.

Construction of the *CPE0897* mutant

A 609-bp DNA fragment was amplified from the coding region of CPE0897 by PCR using an appropriate primer set. The resulting PCR fragment was cloned into the SmaI site of plasmid pUC18 containing the *ermBP* (erythromycin-resistance) gene from *C. perfringens* [26]. The constructed plasmid was electroporated into *C. perfringens* strain 13, and erythromycin-resistant transformants resulting from single-crossover homologous recombination in the *CPE0897* region were selected. The insertion mutation of *CPE0897* was confirmed by PCR and Southern hybridization using a *CPE0897* gene probe. The *CPE0897* mutant function is lost downstream of *CPE0897*.

Construction of the TCS_{eut} (*CPE0895/0896*) mutant strain and complementation with intact *CPE0895/0896* genes

To construct a single-crossover mutant of *CPE0895* (response regulator gene of TCS*eut*), a 519-bp DNA fragment was amplified by PCR using the primers CPE0895F (5'-GTAATAGTCGATGATGAGCCT-3') and CPE0895R (5'-CCATGGTAGTTCTTCTATCC-3'). The PCR fragment was cloned into the SmaI site of plasmid pUC18 containing the ermBP gene [26], and the resulting plasmid was electroporated into *C. perfringens* strain 13. Erythromycin-resistant transformants (*TCSeut* mutant) resulting from single-crossover recombination in the *CPE0895* gene were selected and the mutation was confirmed by PCR and Southern hybridization using a *CPE0895* gene probe.

To clone intact *CPE0895/0896* genes, a chromosomal DNA library of *C. perfringens* strain 13 was constructed as follows. The prepared chromosomal DNA was partially digested with *Hin*dIII. The digested DNA fragments were separated by agarose electrophoresis, and ~ 4.6-kb fragments that might contain *CPE0895/0896* were recovered from the gel, ligated into the *Hin*dIII site of pUC19 and transformed into *E. coli* DH5α. After identification of the plasmid that carried the DNA fragment containing *CPE0895/0896* by Southern hybridization, the 4.6-kb *Hin*dIII fragment was transferred to the *Hin*dIII site of pJIR418. The resulting plasmid was electroporated into the *C. perfringens TCSeut* mutant strain, and the transformants were selected on BHI-Sheep blood agar plates containing 25μg/ml chloramphenicol.

Northern and Southern hybridizations

Total RNA was extracted from *C. perfringens* according to the method described previously. [27] Northern hybridization was also performed, as described previously [24)28)], with the exceptions that DNA fragments were labeled using an AlkPhos-direct kit (GE Healthcare) and signals were detected by CDPstar chemiluminescence (GE Healthcare). DNA probes were prepared by PCR from genomic DNA from *C. perfringens* strain 13 with the appropriate primer sets. Southern hybridization was also performed using the same labeling method and detection procedures.

RESULTS

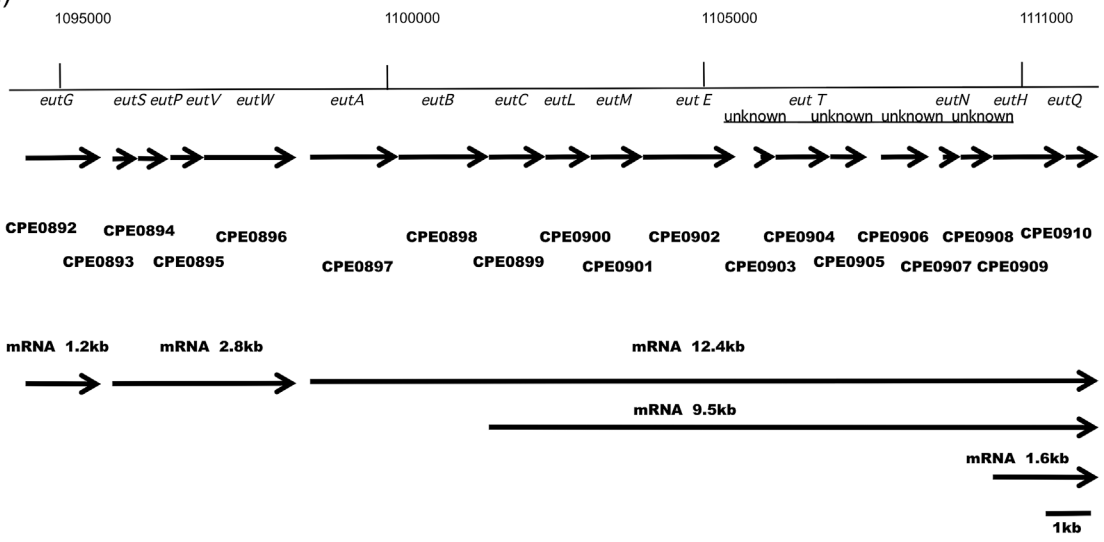
Organization of EA utilization (*eut*) genes in *C. perfringens* strain 13

A bioinformatic analysis of the *C. perfringens* strain 13 genome showed that many of the *eut* genes were found as a cluster in the genome [29]. To investigate the functions of the *eut* genes and their neighboring genes, we searched the nucleotide sequence databases for similarities with known protein databases using a BLAST-search [30]. The results revealed that the *eut*-associated genes in *C. perfringens* were located in a region 16.8 kb in length and consisted of a total of 19 genes (Fig. 1a, 1b). *CPE0895* and *CPE0896* (named TCS_{eut}) encode a response regulator and a sensor histidine kinase of bacterial TCS, respectively. The other 19 genes were found upstream (*eutG*, *-S*, *-P*) or downstream (*eutA, -B, -C, -L, -M, -E, -T, -N, -H, -Q*) of the TCS_{eut} genes.

We compared the amino acid sequences encoded by these genes with those of the eut genes of *S. typhimurium* that have been thoroughly analyzed [7], and found that *C. perfringens* and *S. typhimurium*

share many genes for eut function (Fig. 1b). These data showed that the essential genes for EA metabolism were localized as a cluster in the *C. perfringens* genome. However, the TCS_{eut} genes present in *C. perfringens* were missing in the respective region of *S. typhimurium*, whereas the *eutJ*, *K*, and *R* genes were not found in *C. perfringens*. The ackA gene (CPE1724), encoding an acetate kinase A, and *eutD* gene (*CPE1725*), encoding a phosphotransacetylase were not found within the *eut* region but were located in other chromosomal loci in *C. perfringens*, indicating that the genetic organization of the *eut* genes in *C. perfringens* is quite similar, but slightly different from that in *S. typhimurium*. Recent studies of Gram-positive *E. faecalis* showed that a *TCS* gene is present in the *eut* region instead of *eutR*, just as in the case of *C. perfringens*, and *E. faecalis* TCS was associated with regulation of the transcription of the *eut* operon [7)31)]. These facts suggest that CPE0895/0896 (TCS_{eut}) might be involved in the regulation of transcription of the *eut* genes in *C. perfringens*.

(a)



(b)

Locus in *C.perfringens*	Gene name	Function	Locus in *S. Typhimurium*
CPE 0892	eutG	Alcohol dehydrogenase	STM 2461
CPE 0893	eutS	Carboxysome structural protein	STM 2470
CPE 0894	eutP	Ethanolamine utilization protein	STM 2469
CPE 0895	eutV	Response regulator	unknown
CPE 0896	eutW	Sensor histidine kinase	unknown
CPE 0897	eutA	Reactivating factor	STM 2459
CPE 0898	eutB	EA ammonia lyase large subunit	STM 2458
CPE 0899	eutC	EA ammonia lyase small subunit	STM 2457
CPE 0900	eutL	Carboxysome structural protein	STM 2456
CPE 0901	eutM	Carboxysome structural protein	STM 2465
CPE 0902	eutE	Acetaldehyde dehydrogenase	STM 2463
CPE 0903	unknown		
CPE 0904	eutT	Cobalamin adenosyltransferase	STM 2467
CPE 0905	unknown		
CPE 0906	unknown		
CPE 0907	eutN	Carboxysome structural protein	STM 2464
CPE 0908	unknown		
CPE 0909	eutH	Transport protein	STM2460
CPE 0910	eutQ	Ethanolamine utilization protein	STM 2468
CPE 1724	eutD	Phosphotransacetylase	STM 2466
CPE 1725	ackA	Acetate kinase A	unknown
unknown	eutJ	Heatshock protein	STM 2462
unknown	eutK	Carboxysome structural protein	STM 2455
unknown	eutR	Regulator ethanolamine operon	STM 2454

Fig. 1. (a) Gene organization and transcription map of the *eut* operon in *C. perfringens* strain 13.The genetic organization was drawn from the nucleotide sequence of the *C. perfringens* strain 13 chromosome [24)]. The length and direction of the major transcripts calculated from Northern analyses (shown below) are shown by arrows. (b) Putative function of the product of the *eut* genes in *C. perfringens*, as deduced from the similarity with the eut proteins from *S. typhimurium*.

The *eut* operon has been best studied in *S. typhimurium* [32]. In general, two sequential reactions convert EA into acetyl-CoA (Fig. 2a). First, EA is converted to acetaldehyde by the EA ammonia-lyase (EC 4.3.1.7)(EutB, C) [33]. In the second step, the acetaldehyde is converted to acetate and acetyl-CoA by the acetaldehyde dehydrogenase (EutE) [34]. Acetyl-CoA is subsequently used in various metabolic processes, such as the tricarboxylic acid (TCA) cycle, the glyoxylate cycle or in lipid biosynthesis. Since the TCA cycle does not exist in *C. perfringens*, acetyl-CoA could be converted into acetyl phosphate by phosphotransacetylase (EutD) and further converted into acetate by the housekeeping acetate kinase (AckA), while concomitantly generating ATP (Fig. 2a) [35)36)]. In addition, EutH might be a membrane protein necessary for the transport of EA. EutS, EutM, EutN, EutL and EutK might be shell structural proteins comprising a carboxysome, an organelle required to conserve volatile metabolites (e.g., acetaldehyde) and to concentrate the EA catabolic enzymes [34)-38)].

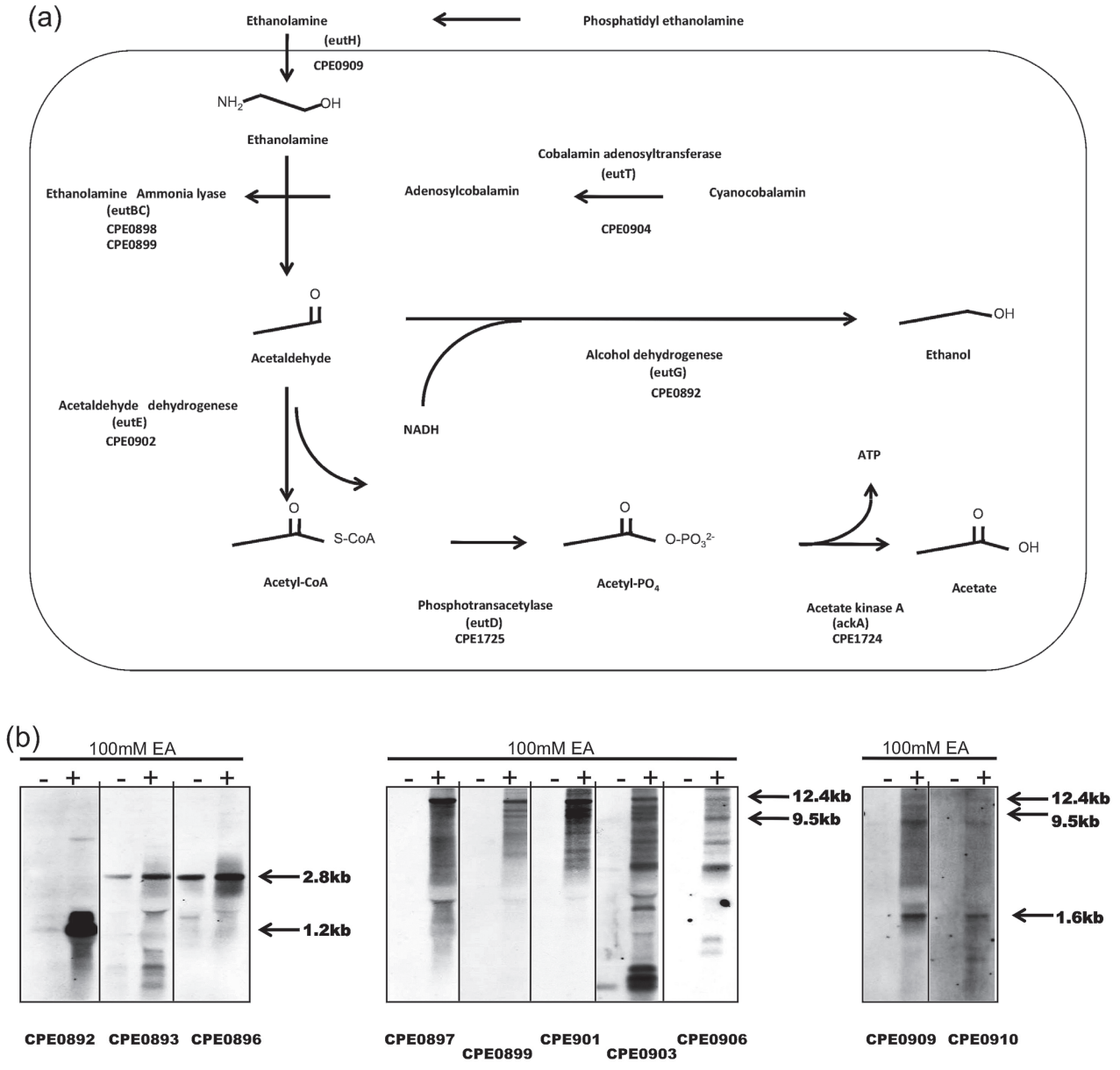


Fig. 2. (a) Pathway of EA metabolism and putative functions of the products of the ethanolamine utilization (eut) operon in *C. perfringens*. (b) Northern-blot analyses of the *eut* operon. The total RNA from wild-type strain 13 was prepared from cultures grown for 2 hr in YT medium (-) only and YT medium supplemented with 100 mM EA (+). Then 20 μg of total RNA was run on 0.8% agarose gel and subjected to Northern hybridization. Representative data from two experiments that showed the same results are presented.

Transcription of the eut operon is induced by extracellular EA

To investigate the operon structure and the effect of extracellular EA on the expression of the putative *eut* genes, we examined their transcription pattern by Northern hybridization using each gene probe, both in the presence and absence of EA in the culture media (Fig. 2b). Consequently, five major transcripts were detected in the *eut* gene region. The probes of *CPE0893-0896*, *CPE0892*, *CPE0897-0910*, *CPE0899-0910*, and *CPE0909-0910* detected 2.8-kb, 1.2-kb, 12.4-kb, 9.5-kb, and 1.6-kb mRNAs, respectively, all of which were apparently induced by the addition of 100 mM EA to the media. These results clearly indicated that the *eut* genes in *C. perfringens* are transcribed as multiple mRNAs and that their expression is induced by the presence of extracellular EA.

***C. perfringens* can utilize EA as an energy source**

To examine the role of the *eut* operon on the growth of *C. perfringens*, we cultured the cells in minimal medium under various conditions and compared their growth. There is a broad range in the replication potential of *Clostridium perfringens*. Due to differences in the growth temperature and humidity, the growth of bacteria can vary significantly throughout the day. The data of 4 independent experiments is shown in Fig.3. The wild-type strain 13 in minimal medium containing 0.1% glucose reached the stationary phase at 11 to 13 hrs after the start of culture. When 100 mM EA was added, the wild-type strain 13 reached the stationary phase after 9 to 11 hrs of culturing, indicating that it grew faster in the medium containing 100 mM EA than in the medium containing 0.1% glucose alone. In contrast, the *CPE0897* mutant reached the stationary phase after 12 to 15 hrs of culture or later, regardless of whether or not 100 mM EA was added, indicating that the *CPE0897* mutant grew more slowly than the wild type. In these four independent experiments it was consistently found that the wild-type strain 13 grew faster in the medium containing 100 mM EA compared with the other strains. These data supposed that the presence of the *eut* operon positively affects the growth of *C. perfringens*, and that the *eut* operon might be involved in energy

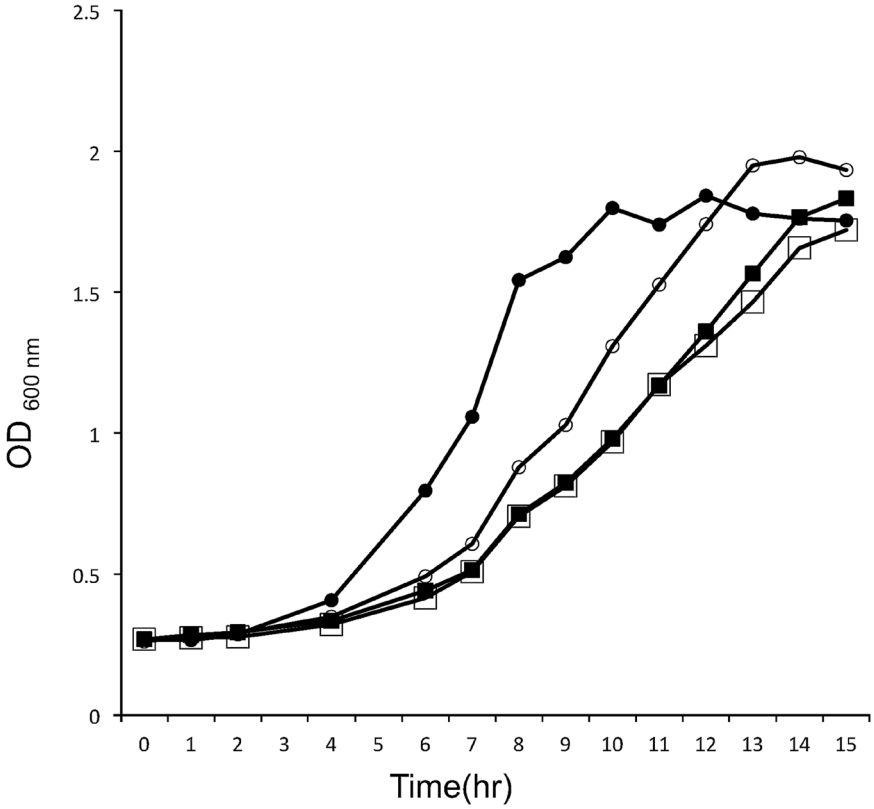


Fig. 3. Growth curves of *C. perfringens* strains. Growth curves of the wild type (circles) and CPE0897 mutant (squares) are shown. Each strain was grown in minimum medium containing 0.1% glucose (open symbols) and minimum medium containing 0.1% glucose and 100 mM EA (closed symbols) (Wild type/-EA; ○ Wild type/+EA; ● Mutant/-EA; □ Mutant/+EA; ■). OD 600 nm was measured at the times indicated. A representative data from four independent experiments is presented.

metabolism, thus contributing to the rapid growth of the organism.

Role of TCS_{eut} in the expression of the *eut* operon

To investigate the genetic regulation of the *eut* operon, we constructed a TCS_{eut} (*CPE0895/0896*) mutant strain by using single crossover homologous recombination. The transcriptional induction of the *eut* operon by EA in the wild-type strain was compared with that in the TCS_{eut} mutant strain by Northern analysis. The expression of 1.2-kb mRNA of *CPE0892* was decreased in the TCS_{eut} mutant strain, while the expression of *CPE0892* was restored by complementation with the intact TCS_{eut} genes (Fig. 4a). All mRNAs of 1.6-kb, 9.5-kb, 12.4-kb were also decreased in the TCS_{eut} mutant strain, although the expression of these transcripts was restored only weakly by complementation with the intact TCS_{eut} gene (Fig. 4b). These data indicated that the TCS_{eut} acts as a transcriptional regulator for the *eut* operon by positively regulating the EA-induced transcription of the *eut* operon.

Transcriptional regulation of the *eut* operon by the VirR/VirS and *virX* system

In *C. perfringens*, previous studies have shown that global regulatory systems, including the two-component VirR/VirS-VR-RNA system and the *virX*

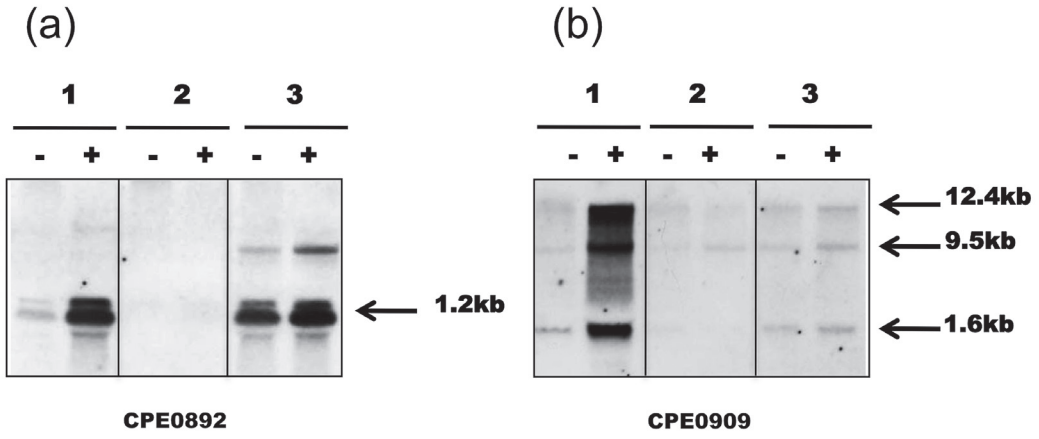


Fig 4. Northern-blot analyses of the TCS_{eut} mutant. Bacteria were cultured in YT medium with appropriate antibiotics at 37℃ under anaerobic conditions. (a) Total RNA was prepared and 20 μg of total RNA was run on 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with the probe for CPE0892. (b) Total RNA under the same conditions hybridized with the probe for CPE0909. The wild-type strain 13 (lane 1), TCS_{eut} mutant strain (lane 2) and TCS_{eut} mutant complemented with intact TCS_{eut} genes (lane 3) were cultured for 2 hr. 100 mM EA was added (+) or not added (-), and after 15 min, total RNA was prepared for Northern analysis. Representative data from triplicate experiments that showed similar results are presented.

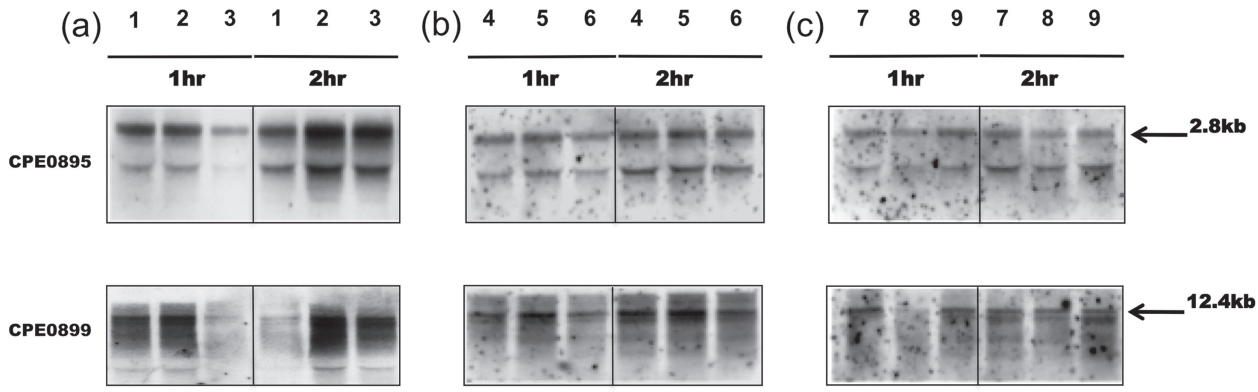


Fig. 5. Northern-blot analyses of the *vir R* mutant (TS 133), *vrr* (VR-RNA) mutant and *VirX* mutant (TS 186). All strains were cultured in YT medium containing 100 mM EA for 1 and 2 hr, and then total RNA was prepared. Either 18 μg (for *vir R* and *vir X*) or 12 μg (for *vrr*) of total RNA was used for the 0.8% agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with probes for *CPE0895*, *CPE0899* and *CPE0909*. The three panels show the results for (a) strain 13 (pJIR418) (lane 1), strain TS133 (pJIR418) (lane 2), and strain TS133 (pBT405) (lane 3); (b) strain 13 (pJIR418) (lane 4), strain TS140 (pJIR418) (lane 5), and strain TS140 (pSB1031) (lane 6); and (c) strain 13 (pJIR418) (lane 7), strain TS186 (pJIR418) (lane 8), and strain TS186 (pTS907) (lane 9). The experiments were repeated at least three times and a representative results is shown.

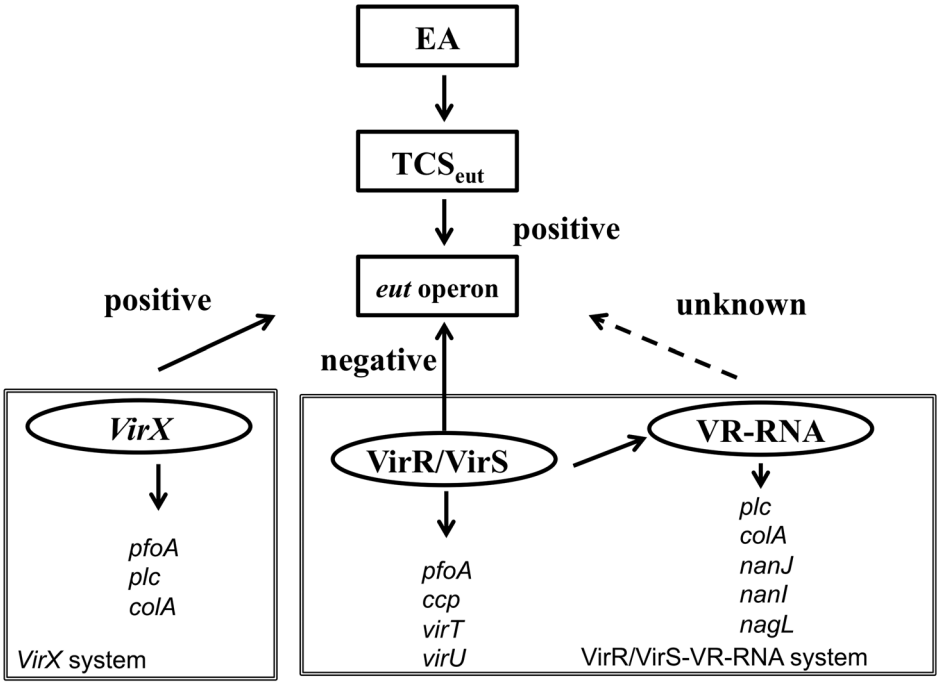


Fig.6. Simplified model of the ethanolamine utilization (eut) operon regulatory network. The *TCS*$_{eut}$ acts as a positive transcriptional regulator for the *eut* operon. VirR/VirS negatively regulates the *eut operon*. *VirX* positively regulates the *eut operon*. The VirR/VirS -VR-RNA system and virX system are shown in the doublet squares.

system, regulate various virulence-related genes, such as *plc*, *colA*, and *pfoA* encoding alpha-toxin, kappa-toxin and theta-toxin, respectively [3)12)]. Moreover, the VirR/VirS-VR-RNA system also controls various genes for nutritional metabolism [17-20)]. Herein, we investigated whether the VirR/VirS-VR-RNA system and/or VirX system are involved in the transcriptional regulation of the *eut* operon.

As shown in Fig. 5a, the 2.8-kb and 12.4-kb *eut* transcripts were increased in the VirR/VirS mutant strain TS133 at 2 hrs, compared with those in the wild-type and *virR/virS*-complemented TS133. These findings indicate that transcription of the *eut* operon might be negatively regulated by the VirR/VirS system. However, the expression of the 2.8-kb and 12.4-kb mRNAs in the VR-RNA mutant strain TS140 was not significantly different from the wild type, under the conditions tested (Fig. 5b). The expression of the 2.8-kb and 12.4-kb mRNAs was decreased in the *virX* mutant strain TS186 after 1 hr, suggesting that *virX* regulates these transcripts positively in the wild type (Fig. 5c). These results indicate that a complex transcriptional regulatory network involving the two-component VirR/VirS and the *virX* system coordinately controls the expression of the *eut* operon in *C. perfringens* (Fig. 6).

DISCUSSION

This study revealed that the *eut* operon in the *C. perfringens* genome might play an important role in the energy metabolism of EA. It has been proposed that the host diet, and the cell membranes of the bacteria and/or epithelial cells in the intestine might be rich sources of EA [6)]. Therefore, these data supposed that *C. perfringens* could grow rapidly in human and animal bodies by utilizing the abundant EA from the host. Since rapid growth in the host is essential to establish gas gangrene, we consider that the *eut* operon may play an important role in the pathogenicity of *C. perfringens*.

The expression of the *eut* operon was locally regulated by the TCS$_{eut}$ upstream of the *eut* operon. In *Salmonella* species, expression of the *eut* operon is positively controlled by a DNA-binding transcription factor, EutR, downstream of the *eut* operon [37)39)]. In contrast, Gram-positive *E. faecalis* lacks the EutR regulator, but has a two-component system (EutV/EutW). The RR (EutV) in *E. faecalis* has an AmiR and NasR transcription antitermination regulators (ANTAR) domain, indicating that it might also bind to RNA and disrupt transcriptional terminators to affect target gene expression [7)31)]. A recent study

suggested that a 13-nucleotide element (AGCAANGRRGCUY) comprises the anti-terminator recognition element and that the binding of EutV to this site in the mRNA prevents terminator formation, resulting in positive regulation of the *eut* operon in *E. faecalis* [31]. The identities of genes related to the binding of EutV remain controversial and the anti-terminator recognition element has been reported to be located upstream of *eutP*, *eutG*, *eutS*, and *eutA* in *E.faecalis*. These observations suggest multiple points of posttranscriptional regulatory control by EutW/V [40]. Therefore, it has been suggested that transcription upstream and downstream of the *eut* operon might occur through variants that are positively regulated by the TCS_{eut} upstream of the *eut* operon of *C. perfringens* and that the TCS_{eut} might play an important role in the control of EA-dependent energy production in *C. perfringens*. The expression of *CPE0892* was restored strongly by complementation with the intact TCS_{eut} gene (Fig. 4a) without EA unlike others expressions(Fig. 4b). It is overexpression because of the expression of *CPE0892* was been weakly by wild-type without EA. And these data indicated that the TCS_{eut} acts as a transcriptional regulator for the *eut* operon at multiple points. Therefore, further studies will be needed to clarify whether EA acts as an activation signal of the histidine kinase (CPE896), thereby leading to induction of the entire *eut* operon.

Moreover, the two-component VirR/VirS system and the *virX* system also affects the expression of the *eut* operon, possibly through control of the 2.8-kb mRNA that encodes a local two-component regulator, TCS_{eut} (CPE0895/0896). Previous studies have reported that the two-component VirR/VirS system also affects transcription of the nutrient metabolism genes in *C. perfringens*: the hemeoxygenase gene for acquiring iron, the acid phosphatase gene for acquiring inorganic phosphate, the *myo*-inositol operon and citrate utilization operon, which are related to various metabolic pathways and are believed to be associated with the pathogenicity of *C. perfringens* [17)-20)]. In this study, it was found that the two-component VirR/VirS system similarly affects transcription of the *eut* gene. We also supposed that *C. perfringens* might grow rapidly using EA as an energy source, in addition to glucose, which would then enable the cells to produce toxins and enzymes that are essential for further nutritional uptake and the development of gas gangrene in the human body. Therefore, it would be reasonable to conclude that the *eut* operon plays important roles in the pathogenicity of *C. perfringens*.

REFERENCE LIST

1) McDonel JL. Clostridium perfringens toxins (type A, B, C, D, E). Pharmacol Ther 10: 617-655, 1980

2) Smith LD. Virulence factors of Clostridium perfringens. Rev Infect Dis 1: 254-262, 1979

3) Rood JI. Virulence genes of Clostridium perfringens. Annu Rev Microbiol 52: 333-360, 1998

4) Randle CL, Albro PW, Dittmer JC. The phosphoglyceride composition of Gram-negative bacteria and the changes in composition during growth. Biochim Biophys Acta 187: 214-220, 1969

5) Blackwell CM, Scarlett FA, Turner JM. Ethanolamine catabolism by bacteria, including Escherichia coli. Biochem Soc Trans 4: 495-497, 1976

6) Chang GW, Chang JT. Evidence for the B12-dependent enzyme ethanolamine deaminase in Salmonella. Nature 254: 150-151, 1975

7) Del Papa MF, Perego M. Ethanolamine activates a sensor histidine kinase regulating its utilization in Enterococcus faecalis. J Bacteriol 190: 7147-7156, 2008

8) Korbel JO, Doerks T, Jensen LJ, Perez-Iratxeta C, Kaczanowski S, Hooper SD, Andrade MA, Bork P. Systematic association of genes to phenotypes by genome and literature mining. PLoS Biol 3: e134, 2005

9) Jung K. Fried L, Behr S, Heermann R. Histidine kinases and response regulators in networks. Curr Opin Microbiol. 15(2): 118-24, 2012

10) Hoch JA, Silhavy TJ. Two-component signal transduction. Washington, DC: American Society for Microbiology, 1995

11) Lyristis M, Bryant AE, Sloan J, Awad MM, Nisbet IT, Stevens DL, Rood JI. Identification and molecular analysis of a locus that regulates extracellular toxin production in Clostridium perfringens. Mol Microbiol 12: 761-777, 1994

12) Ohtani K, Bhowmik SK, Hayashi H, Shimizu T. Identification of a novel locus that regulates expression of toxin genes in Clostridium perfringens. FEMS Microbiol Lett 209: 113-118, 2002

13) Shimizu T, Ba-Thein W, Tamaki M, Hayashi H. The virR gene, a member of a class of two-component response regulators, regulates the production of perfringolysin O, collagenase, and hemagglutinin in Clostridium perfringens. J Bacteriol 176: 1616-1623, 1994

14) Shimizu T, Yaguchi H, Ohtani K, Banu S, Hayashi H. Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. Mol Microbiol 43: 257-265, 2002

15) Ohtani K, Hirakawa H, Tashiro K, Yoshizawa S, Kuhara S, Shimizu T. Identification of a two-component VirR/VirS regulon in Clostridium perfringens. Anaerobe 16: 258-264, 2010

16) Okumura K, Ohtani K, Hayashi H, Shimizu T.

Characterization og genes regulated directly be the VirR/VirS system in clostridium perfringens. J Bacteriol. 190(23): 7719-27, 2008
17) Hassan S, Ohtani K, Wang R, Yuan Y, Wang Y, Yamaguchi Y, Shimizu T. Transcriptional regulation of hemO encoding heme oxygenase in Clostridium perfringens. J Microbiol 48: 96-101, 2010
18) Kawsar HI, Ohtani K, Okumura K, Hayashi H, Shimizu T. Organization and transcriptional regulation of myo-inositol operon in Clostridium perfringens. FEMS Microbiol Lett 235: 289-295, 2004
19) Wang R, Ohtani K, Wang Y, Yuan Y, Hassan S, Shimizu T. Genetic and biochemical analysis of a class C non-specific acid phosphatase (NSAP) of Clostridium perfringens. Microbiology 156: 167-173, 2010
20) Yuan Y, Ohtani K, Yoshizawa S, Shimizu T. Complex transcriptional regulation of citrate metabolism in Clostridium perfringens. Anaerobe 18: 48-54, 2012
21) Ohtani K, Hirakawa H, Paredes-Sabja D, Tashiro K, Kuhara S, Sarker MR, Shimizu T. Unique regulatory mechanism op sporulation and enterotoxin production in clostridium perfringens. J Bacteriol. 195(12): 2931-6, 2013
22) Mahony DE, Moore TI. Stable L-forms of Clostridium perfringens and their grouth on glass surfaces. Can J Microbiol. 22(7): 953-9, 1976
23) Sloan J, Warner TA, Scott PT, Bannam TL, Berryman DI, Rood JI. Construction of a sequenced Clostridium perfringens-Escherichia coli shuttle plasmid. Plasmid 27: 207-219, 1992
24) Ba-Thein W, Lyristis M, Ohtani K, Nisbet IT, Hayashi H, Rood JI, Shimizu T. The virR/virS locus regulates the transcription of genes encoding extracellular toxin production in Clostridium perfringens. J Bacteriol 178: 2514-2520, 1996
25) Riha WEJr, Solberg M. Chemically defined medium for the growth of Clostridium perfringens. Appl Microbiol 22: 738-739, 1971
26) Rood JI, Cole ST. Molecular genetics and pathogenesis of Clostridium perfringens. Microbiol Rev 55: 621-648, 1991
27) Aiba H, Adhya S, de Crombrugghe B. Evidence for two functional gal promoters in intact Escherichia coli cells. J Biol Chem 256: 11905-11910, 1981
28) Kobayashi T, Shimizu T, Hayashi H. Transcriptional analysis of the beta-galactosidase gene (*pbg*) in Clostridium perfringens. FEMS Microbiol Lett 133: 65-69, 1995
29) Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, Ogasawara N, Hattori M, Kuhara S, Hayashi H. Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. Proc Natl Acad Sci USA 99: 996-1001, 2002
30) Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 215: 403-410, 1990
31) Fox KA, Ramesh A, Stearns JE, Bourgogne A, Reyes-Jara A, Winkler WC, Garsin DA. Multiple posttranscriptional regulatory mechanisms partner to control ethanolamine utilization in Enterococcus faecalis. Proc Natl Acad Sci U S A 106: 4435-4440, 2009
32) Brinsmade SR, Paldon T, Escalante-Semerena JC. Minimal functions and physiological conditions required for growth of Salmonella enterica on ethanolamine in the absence of the metabolosome. J Bacteriol 187: 8039-8046, 2005
33) Faust LR, Connor JA, Roof DM, Hoch JA, Babior BM. Cloning, sequencing, and expression of the genes encoding the adenosylcobalamin-dependent ethanolamine ammonia-lyase of Salmonella typhimurium. J Biol Chem 265: 12462-12466, 1990
34) Stojiljkovic I, Baumler AJ, Heffron F. Ethanolamine utilization in Salmonella typhimurium: nucleotide sequence, protein expression, and mutational analysis of the cchA cchB eutE eutJ eutG eutH gene cluster. J Bacteriol 177: 1357-1366, 1995
35) Brinsmade SR, Escalante-Semerena JC. The eutD gene of Salmonella enterica encodes a protein with phosphotransacetylase enzyme activity. J Bacteriol 186: 1890-1892, 2004
36) Starai VJ, Garrity J, Escalante-Semerena JC. Acetate excretion during growth of Salmonella enterica on ethanolamine requires phosphotransacetylase (EutD) activity, and acetate recapture requires acetyl-CoA synthetase (Acs) and phosphotransacetylase (Pta) activities. Microbiology 151: 3793-3801, 2005
37) Penrod JT, Mace CC, Roth JR. A pH-sensitive function and phenotype: evidence that EutH facilitates diffusion of uncharged ethanolamine in Salmonella enterica. J Bacteriol 186: 6885-6890, 2004
38) Sheppard DE, Penrod JT, Bobik T, Kofoid E, Roth JR. Evidence that a B12-adenosyl transferase is encoded within the ethanolamine operon of Salmonella enterica. J Bacteriol 186: 7635-7644, 2004
39) Roof DM, Roth JR. Ethanolamine utilization in Salmonella typhimurium. J Bacteriol 170: 3855-3863, 1988
40) Garsin DA. Ethanolamine utilization in bacterial pathogens: roles and regulation. Nat Rev Microbiol. 8(4): 290-5, 2010