厚生労働科学研究費補助金
食品の安心・安全確保推進研究事業

# ウェルシュ菌芽胞形成調節ネットワークの解析と、調節遺伝子をターゲットとした食中毒予防法の開発に関する研究

平成21年度　総括研究報告書

研究代表者　大谷　郁

平成22（2010）年5月

厚生労働科学研究費補助金

食品の安心･安全確保推進研究事業

# ウェルシュ菌芽胞形成調節ネットワークの解析と、調節遺伝子をターゲットとした食中毒予防法の開発　に関する研究

平成２１年度　総括研究報告書

研究代表者　大谷　郁

平成２２（２０１０）年　５月

目　　次

厚生労働科学研究費補助金（食品の安心·安全確保推進研究事業）
総括研究報告書

# ウェルシュ菌芽胞形成調節ネットワークの解析と、調節遺伝子をターゲットとした食中毒予防法の開発　に関する研究

研究代表者　　大谷　郁　　金沢大学医薬保健研究域医学系　講師

研究要旨
ウェルシュ菌食中毒は、ウェルシュ菌があらゆる環境に耐えうるための芽胞を形成する時に惹起されると考えられている。しかし、その詳細なメカニズムは明らかになっておらず、ウェルシュ菌の食中毒を予防することは困難である。我々は、食中毒予防法の手がかりをつかむため、腸管毒素産生と芽胞形成メカニズムを遺伝子レベルで明らかとすることを試みた。昨年度マイクロアレイ解析より明らかとなった新規芽胞形成調節遺伝子による芽胞形成調節ネットワークの詳細について解析した。この新規調節遺伝子は、芽胞形成に関与するシグマ因子の転写を負に調節することで、芽胞形成を負に調節していることが明らかとなった。また、新規調節遺伝子の転写を制御する二成分制御系の存在が明らかとなった。

## A. 研究目的

グラム陽性嫌気性桿菌ウェルシュ菌は、日本において食中毒の主要原因菌の１つとしてあげられる。この食中毒は、本菌が加熱不十分な食べ物とともに人体に入り、胃酸の刺激により芽胞を形成し、芽胞形成中に下痢を引き起こす腸管毒素（エンテロトキシン）を産生することが原因であると報告されている。しかし、その詳細なメカニズム、特に芽胞形成と腸管毒素産生の制御機構については未知のままである。本菌の食中毒予防法としては食物をよく加熱することしかないのが現状であり、大量に食べ物を調理する学校給食や仕出し弁当等での食中毒は未だに回避することが難しい状態である。そこで、昨年度同定した新規芽胞形成調節遺伝子による芽胞形成ネットワークの詳細を明らかとし、遺伝子レベルで芽胞をコントロールし、食中毒予防につなげることを本研究の目的とした。

B. 研究方法

昨年度同定した調節遺伝子の変異株をガス壊疽株、食中毒由来株の両株を用いて作製し、その遺伝子発現をノザン解析、リアルタイムPCRを用いて、芽胞形成効率をコロニーカウント法または顕微鏡により確認した。芽胞形成に関与する二成分制御系の同定には、二成分制御系全変異株を用いて、ノザン解析によりスクリーニングを行った。

C. 研究結果

新規芽胞形成調節遺伝子の変異株をガス壊疽株ならびに食中毒由来株を用いて複数作製、またその遺伝子相補株も作製した。これらの株の解析により、食中毒由来株、ガス壊疽株のどちらも変異株で野生株に比べて芽胞形成効率が上昇し、相補株で減少した。この結果より、この調節遺伝子は芽胞形成を負に調節していることが明らかとなった。さらに、変異株、相補株における芽胞形成関連遺伝子の発現をノザン解析により確認した結果、変異株においては、芽胞形成に関与することがすでに報告されているシグマ因子の転写やその他の芽胞形成関連遺伝子の転写も負に調節していることが明らかとなった。通常今回用いたガス壊疽株は芽胞形成の鍵となると考えられていた *spo0A* 遺伝子に変異があるため、芽胞形成がおこらないが、新規調節遺伝子変異株では芽胞形成が行われることから、この調節遺伝子は、*spo0A* の転写も調節するが、その下位に存在するシグマ因子の転写も *spo0A* を介さずに調節できることが明らかとなった。さらに、全二成分制御系遺伝子破壊株のスクリーニングにより、新規調節遺伝子の制御に関与する少なくとも３つの二成分制御系遺伝子が存在することが明らかとなった。

D. 考察

これらの結果は、新規調節遺伝子が、芽胞形成を包括的に調節する調節遺伝子であることを示している。また、この調節遺伝子は調節 RNA として機能することを既に明らかとしており、この調節遺伝子を含む芽胞形成調節新規ネットワークをさらに詳細に解析することは、RNA をターゲットとしたウェルシュ菌食中毒予防法の開発の大きな手がかりとなり得ると考えられる。さらには今回明らかとなった二成分制御系の感知するシグナルを同定できれば、このシグナルをターゲットとした対策も可能になってくると考えられる。

E. 結論

新規調節遺伝子は芽胞形成を転写レ

ベルで包括的に調節していることが強く示唆されたこと、また、その調節遺伝子の転写制御に関与するに成分制御系の存在が明らかになったことは、本菌の食中毒は食品に混入した菌が体内に入る時に芽胞になり、腸管毒素を産生することで食中毒が発生することを考えると、この新規調節ネットワークをさらに解析することは、食中毒予防法開発に新たな側面を与える可能性が考えられる。

F. 健康危険情報

特になし

G. 研究発表

1. 論文発表

1. Kaori Ohtani, Hideki Hirakawa, Kousuke Tashiro, Satoko Yoshizawa, Satoru Kuhara, Tohru Shimizu. 2010. Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*. Anaerobe. (in press)

2. Hassan S, Ohtani K, Wang R, Yuan Y, Wang Y, Yamaguchi Y and Shimizu T. 2010. Journal of Microbiology. Transcriptional regulation of hemO encoding heme oxygenase in *Clostridium perfringens*. 48(1):96-101.

3. Ohtani K, Yuan Y, Hassan S, Wang R, Wang Y, and Shimizu T 2009. Virulence gene regulation by the *agr* system in *Clostridium perfringens* J. Bacteriol. 191(12): 3919-3927)

4. Vidal JE, Ohtani K, Shimizu T, McClane BA 2009. Contact with enterocyte-like Caco-2 cells induces rapid upregulation of toxin production by *Clostridium perfringens* type C isolates. Cell Microbiol. 11(9):1306-28.

2. 学会発表

1. Kaori Ohtani, Yonghui Yuan, Yumi Yamaguchi, Satoko Yoshizawa, Etab Abo-Remala, Tohru Shimizu
Gene Regulation by multiple signals in *Clostridium perfringens*.
日本細菌学雑誌 2009 64-1: 80

2. Kaori Ohtani, Y. Yuan, H Sufi, R. Wang, Y. Wang and Tohru Shimizu
Gene regulation by intra-and inter-cellular signaling in *Clostridium perfingens*.
6th Clostpath international conference

研究成果の刊行に関する一覧表

雑誌

| 発表者氏名 | 論文タイトル名 | 発表誌名 | 巻号 | ページ | 出版年 |
|---|---|---|---|---|---|
| Kaori Ohtani, Hideki Hirakawa, Kousuke Tashiro, Satoko Yoshizawa, Satoru Kuhara, Tohru Shimizu | Identification of a two-component VirR/VirS regulon in *Clostridium perfringens* | Anaerobe | in press | | 2010 |
| Hassan S, Ohtani K, Wang R, Yuan Y, Wang Y, Yamaguchi Y and Shimizu T | Transcriptional regulation of hemO encoding heme oxygenase in *Clostridium perfringens*. | Journal of Microbiology | 48(1) | 96-101 | 2010 |
| Ohtani K, Yuan Y, Hassan S, Wang R, Wang Y, and Shimizu T | Virulence gene regulation by the *agr* system in *Clostridium perfringens* | Journal of Bacteriology | 191(12) | 3919-3927 | 2009 |
| Vidal JE, Ohtani K, Shimizu T, McClane BA | Contact with enterocyte-like Caco-2 cells induces rapid upregulation of toxin production by *Clostridium perfringens* type C isolates. | Cell Microbiol | 11(9) | 1306-1328 | 2008 |

Anaerobe xxx (2009) 1–7

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Molecular Biology, Genetics and Biotechnology

# Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*

Kaori Ohtani [a], Hideki Hirakawa [b], Kousuke Tashiro [b], Satoko Yoshizawa [a], Satoru Kuhara [b], Tohru Shimizu [a,*]

[a] Department of Bacteriology, Graduate School of Medical Science, Kanawaza University, 13-1, Takara-Machi Kanazawa, Ishikawa 920-8640, Japan
[b] Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

ARTICLE INFO

*Article history:*
Received 28 June 2009
Received in revised form
1 September 2009
Accepted 8 October 2009
Available online xxx

*Keywords:*
Microarray
Two-component system
Pathogenesis

ABSTRACT

*Clostridium perfringens*, a Gram-positive anaerobic pathogen, is a causative agent of human gas gangrene that leads to severe rapid tissue destruction and can cause death within hours unless treated immediately. Production of several toxins is known to be controlled by the two-component VirR/VirS system involving a regulatory RNA (VR-RNA) in *C. perfringens*. To elucidate the precise regulatory network governed by VirR/VirS and VR-RNA, a series of microarray screening using VirR/VirS and VR-RNA-deficient mutants was performed. Finally, by qRT-PCR analysis, 147 genes (30 single genes and 21 putative operons) were confirmed to be under the control of the VirR/VirS-VR-RNA regulatory cascade. Several virulence-related genes for alpha-toxin, kappa-toxin, hyaluronidases, sialidase, and capsular polysaccharide synthesis were found. Furthermore, some genes for catalytic enzymes, various genes for transporters, and many genes for energy metabolism were also found to be controlled by the cascade. Our data indicate that the VirR/VirS-VR-RNA system is a global gene regulator that might control multiple cellular functions to survive and multiply in the host, which would turn out to be a lethal flesh-eating infection.



## 1. Introduction

*Clostridium perfringens* is a Gram-positive, spore-forming anaerobic bacterium, and the type A strain is recognized as a major pathogen in humans [13,24]. Numerous toxins and enzymes that the organism secretes are thought to contribute to the pathogenicity of fatal infections (gas gangrene or myonecrosis) [25]. Although the toxins and enzymes have their specific activities and roles in the disease process, their synergistic actions on the host tissue are needed for the infection [2,25]. Therefore, knowledge of the global regulatory mechanisms of such virulence genes is a prerequisite for understanding the pathogenicity of *C. perfringens*.

Two-component *virR/virS* genes encode the key positive regulators of the production of alpha-toxin, theta-toxin, kappa-toxin, protease, and sialidase in *C. perfringens* [16,28], and the VirR/VirS system positively regulates the transcription of the *plc* (encoding alpha-toxin or phospholipase C) and *colA* (encoding kappa-toxin or collagenase) genes, as well as *pfoA* (encoding theta-toxin or perfringolysin O) [3]. Macroarray experiments identified the VirR/VirS-positively regulated genes (*ptp*, *cpd*, *hyp7*, and *hyp23*) and negatively regulated ones (*metB–cysK–ygaG*), suggesting that the VirR/VirS system regulates not only virulence genes but also many housekeeping genes [4].

Further studies showed that the VirR/VirS-regulated *vrr* (renamed from *hyp7*) gene appears to positively regulate the transcription of *plc* and *colA* but not that of *pfoA* [31]. Deletion studies of the *vrr* region suggested that the *vrr* protein-coding region was not necessary for regulatory function, indicating that the regulatory activity was derived from the transcript itself (named VR-RNA) functioning as a regulatory RNA molecule [31].

Analysis of the complete genomic sequence of *C. perfringens* strain 13 [29] resulted in the identification of five genes (*pfoA* (CPE0162), *virT* (CPE0845), *ccp* (CPE0846), *virU* (CPE0920) and *vrr* (CPE0957)) with consensus VirR-binding sequences [6] upstream of the open reading frame [23]. Transcription of *virT*, *ccp* (encoding alpha-clostripain) and *virU* was found to be positively regulated by the VirR/VirS system just in the same manner with *pfoA* and *vrr*. Genetic analyses revealed that *virT* has a negative effect on expression of *pfoA* and *ccp*, whereas *virU* positively affects expression of *pfoA*, *virT*, *ccp*, and *vrr*. Mutational analyses suggested that *virT* and *virU* may encode RNA regulators rather than proteins, implying that a complex regulatory network involving several regulatory RNA molecules governs the expression of the VirR/VirS-VR-RNA regulon in *C. perfringens* [23].

Genomic features of *C. perfringens* suggest that the organism needs to secrete toxins and enzymes to degrade macromolecules in

* Corresponding author. Tel.: +81 76 265 2200, fax: +81 76 234 4230.
*E-mail address:* tshimizu@med.kanazawa-u.ac.jp (T. Shimizu).

1075-9964/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.anaerobe.2009.10.003

Please cite this article in press as: Ohtani K, et al., Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*, Anaerobe (2009), doi:10.1016/j.anaerobe.2009.10.003

host tissues, after which these small nutrients are imported for the bacteria to use for cellular metabolism and biosynthesis. In humans, this activity would lead to severe gangrenous infections. In this study, we performed a series of microarray screening and qRT-PCR analysis using total RNAs from the wild type, a *virR* mutant, and a *vrr* mutant, which elucidated the global regulatory networks governing multiple cellular functions in *C. perfringens*.

## 2. Materials and methods

### 2.1. Bacterial strains, culture conditions, and RNA preparation

*C. perfringens* strains 13 [17], TS133 (*virR*-null mutant constructed by allelic exchange homologous recombination) [28], and TS140 (*vrr*-null mutant constructed by allelic exchange homologous recombination) [31] were cultured in GAM (Gifu Anaerobic Medium, Nissui, Japan) as described previously. Total RNA from *C. perfringens* was prepared as described elsewhere [28].

### 2.2. Microarray construction

Primers for amplifying the internal DNA fragments of 2660 and 63 ORFs on chromosome and plasmid, respectively, of *C. perfringens* strain 13 were selected by a Blast search program [1]. A PCR using EX Taq (Takara, Japan) against total DNA from strain 13 was performed, and the resulting DNA was spotted onto slide glasses (Matsunami Glass, Japan).

### 2.3. Synthesis of Cy-labeled cDNA probe

Fluorescently-labeled cDNA from total RNA was prepared essentially as described previously [27]. The reaction mixture (40 μl), consisting of 15 μg of total RNA from *C. perfringens*, 2 μg of random primers ($d(N)_6$ and $d(N)_9$, Takara, Japan), 1× power script reaction buffer, 5 mM dATP, 5 mM dCTP, 5 mM dGTP, 2 mM dTTP, 3 mM amino-allyl dUTP (Sigma, USA), 0.01 M DTT, 0.1 μl of RNase inhibitor (TakaRa, Japan), and 2 U of Powerscript reverse transcriptase (BD Biosciences, USA), was incubated at 42 °C for 90 min. The RNA template was degraded by the addition of EDTA and NaOH and heating at 65 °C for 30 min. The fluorescently-labeled cDNA was purified with a Microcon-30 (Millipore, USA). The cDNA solution was incubated with mono-reactive Cy3 or Cy5 dyes (GE Healthcare, USA) at RT for 60 min. Unreacted Cy-dye was removed with $Na_2OH$, and the labeled product was purified by a QIAquick column (Qiagen, Germany).

### 2.4. Microarray hybridization and data analysis

Hybridization was performed essentially as described by DeRisi et al. [11]. Equal volumes of Cy3- and Cy5-labeled cDNA probe were mixed and hybridized to microarray in 5× SSC and 0.5% SDS at 60 °C overnight. The slide was washed three times each with 2× SSC-0.2% SDS at RT, 0.2× SSC-0.2% SDS at 60 °C, amd 0.2× SSC-0.2% SDS at RT, in 0.2× SSC with gentle agitation. Finally, the slide was dried by brief centrifuge. The slide was scanned by an FLA-8000 microarray scanner (Fuji Film, Japan). Each spot density was measured by ArrayVision 6.0 (Imaging Research, USA), and normalization/statistical data analysis was performed with GeneSpring GX 7.3 (Agilent Technologies, USA).

### 2.5. Quantitative reverse-transcription PCR (qRT-PCR)

qRT-PCR was performed using a SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen, USA) with SYBR Green, according to the manufacturer's instructions. Appropriate primer sets were selected by Primer Express (Applied Biosystems, USA). Ten-fold serial dilutions of cellular 16S rRNA were used for control templates to quantify mRNA. A 7300 Real Time PCR System (Applied Biosystems) was used for the quantification of mRNA.

### 2.6. Data deposition

The microarray data used in this study have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE12833 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12833).

## 3. Results and discussion

### 3.1. Transcriptional analysis by DNA microarray

In this study, we used a custom DNA microarray that contained PCR-generated DNA from 2660 genes on the chromosome and 63 genes on the plasmid pCP13, and 144 intergenic regions of *C. perfringens* strain 13 [29]. We compared each chromosomal gene's transcription level between the wild type strain 13 (WT) and the *virR*-mutant strain TS133 [28] or *vrr*-mutant strain TS140 [31]. We also tried to analyze transcriptional profile in pCP13 and intergenic regions, which failed probably due to the low expression levels of transcripts from these regions.

In the microarray screening, we compared the difference in gene expression at three time points covering early-, mid-, and late-exponential phases (i.e., 1.5, 2, and 2.5 h from the start of the culture, respectively) between WT and TS133, or between WT and TS140. The microarray experiments were performed three times on each time point, and triplicate data sets were statistically analyzed by GeneSpring GX software. To find the gene whose mean log2 expression ratio is significantly different from the all genes, we performed the Welch *t*-test intending for use with two samples having possibly unequal variances. *T*-values are calculated for each gene, and *p*-values are directly calculated from the theoretical t-distribution based on the gene's calculated *t*-value. Significantly differentially expressed genes were selected by using a *p*-value threshold of 0.05. Finally, the log2 expression ratio was standardized to normal distribution. The genes showing significant differences in expression ($>2.5\ \sigma$ or $<-2.5\ \sigma$) at any time point of 1.5 h, 2 h, and/or 2.5 h were picked up. In these WT-versus-mutant screening, 171 genes apparently showed differences in expression levels (see Table S1 in the supplementary material).

The 171 genes picked up from this screening could be divided into two groups: one showed significant expression differences, mainly in TS133 and not in TS140 (affected only by VirR/VirS), while the other showed similar expression profiles in both mutant strains (affected by the cascade of VirR/VirS and VR-RNA) (Table S1).

### 3.2. Genes affected by VirR/VirS but not by VR-RNA

The two-component VirR/VirS system has been reported to regulate many genes in *C. perfringens* both positively and negatively, and the regulatory RNA molecule, VR-RNA, is also involved in the transcriptional regulation of toxin genes and other genes as a secondary regulator of the VirR/VirS system [3,4,21,22,30,31]. In this microarray screening, *pfoA*, *virT*, *ccp*, and *vrr*, which encode perfringolysin O (theta-toxin), a hypothetical 22.9 kDa protein, alpha-clostripain, and VR-RNA, respectively, were found to be affected only by the VirR/VirS system (Table S1). These four genes have consensus VirR-binding sites upstream from their promoters [29] and were proven to be positively regulated at the transcriptional level through the direct binding of the VirR protein to their VirR-binding sites [5,6,23], which would validate our microarray

Please cite this article in press as: Ohtani K, et al., Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*, Anaerobe (2009), doi:10.1016/j.anaerobe.2009.10.003

results. However, another VirR/VirS-regulated gene, *virU* (CPE0920) [23] did not satisfy the selection criteria, since its expression level was very low.

### 3.3. Genes influenced by VirR/VirS-VR-RNA cascade

In contrast to the small number of genes directly affected by VirR/VirS, we found that expression of 167 genes appeared to be affected by the VirR/VirS-VR-RNA (VirRS-VR) cascade in *C. perfringens*. To further confirm their expression profiles, a quantitative reverse-transcription PCR (qRT-PCR) was performed on each single gene or the first gene of each possible operon (also middle gene in long one). The qRT-PCR analysis was done at three different time points (1.5, 2.0, and 2.5 h) and in triplicate on the total RNA from the cultures of WT, TS140 (*vrr* mutant), and TS140 (pSB1031, *vrr*-complementation plasmid) (Table 1 ). Selecting genes whose mRNA amount in TS140 were >2-fold or <1/2-fold relative to that in wild type, 147 genes were confirmed to be affected by the VirRS-VR cascade; these consisted of 30 single genes and 21 putative operons (Table 1). The other genes listed in Table S1 showed no significant difference and/or no *vrr*-complementation effects in TS140 (pSB1032) by qRT-PCR analysis.

### 3.4. Virulence-associated genes

In addition to the putative virulence genes such as *pfoA* and *ccp* affected by VirR/VirS alone, the VirRS-VR regulatory cascade appeared to influence many virulence-associated genes. Microarray experiments showed that the expression of *plc* and *colA* was positively affected by the VirRS-VR cascade throughout the log phase, since their mRNA amount in TS140 was much less than that in wild type (Table 1). This corresponds well with previous findings that transcription of *plc* and *colA* was positively regulated by VR-RNA at the exponential phase [4,31]. The *plc* gene encodes phospholipase C (lecithinase), which plays a major role in the pathogenesis of gas gangrene [25]. The *colA* gene encodes collagenase, which is thought to degrade collagens in the connective tissue and might help the organism to use the degraded peptides and/or amino acids as nutrients.

Hyaluronidase gene (*nagL*) seemed to be positively influenced by the cascade (Table 1). Hyaluronidase is thought to act as one of the spreading factors that enable tissue-invading bacteria to spread into deeper niches by degrading highly viscous polysaccharides like hyaluronic acids in the connective tissue [14,18]. Moreover, disaccharides, the final products, can be transported and metabolized in the cells to supply the nutrients for replication and spread [14].

Other possible virulence-associated genes, *nanI* (CPE0725, large sialidase) and *nanJ* (CPE0553, very large sialidase) were affected positively at the early- to mid-exponential phases by the cascade (Table 1). Bacterial sialidases have been thought to be virulence-associated enzymes that degrade sialic-acid conjugates in human cells and tissues [8]. The removal of sialic acids from cultured cells by sialidase increases the sensitivity of cells to a membrane-damaging alpha-toxin [12]. Sialidases are also believed to contribute to the uptake of carbon from sources in nature and the human body [8].

Furthermore, the VirRS-VR cascade influenced the expression of the *nanE–nanA* operon (CPE0184-CPE0185) (Table 1). It has been reported that NanE (N-acetylmannosamine-6-phosphate epimerase) and NanA (sialic-acid lyase) are involved in the utilization of sialic acids as energy sources [33]. It is highly possible that enzymes from the *nanE* operon contribute to the generation of energy from small-molecular-weight sialic acids that are available through the degradation of extracellular large-molecular-weight sialic acids by the secreted sialidases. The VirRS-VR cascade might facilitate this metabolic process by its coordinated control of these sialic-acid-related genes.

Three putative virulence operons involved in capsular polysaccharide biosynthesis (CPE0474–CPE0490, CPE491–CPE497 and CPE0500–CPE0502) was under negative control of the VirRS-VR cascade during the exponential phases (Table 1). These putative operons are part of the large capsular gene cluster that is thought to encode for a virulence factor of foreign origin [19]. A capsular polysaccharide associated with *C. perfringens* has been implicated for involvement in its pathogenicity [20]. The mannose-containing capsule is required for binding *C. perfringens* to the surface of J774-33 cells, which enables phagocytosis and escape from the phagosome of macrophages, resulting in the persistent survival of *C. perfringens* in the cytoplasm. This negative tuning of the capsular operons might be involved in the establishment of gas gangrene in the muscular and connective tissue during exponential phase.

### 3.5. Enzymes

The VirRS-VR cascade also affected the expression of various genes encoding putative enzymes. CPE0201, probably encoding acid phosphatase, was positively affected by this cascade throughout the exponential phase (Table 1). Bacterial acid phosphatases are widespread enzymes found in several bacterial taxa, and they function in scavenging organic phosphoesters, degrading various substrates into membrane-permeable inorganic phosphates (Pi) that are essential nutrients for bacteria [26].

The expression of *cpdC* encoding 2′,3′-cyclic-nucleotide 2′-phosphodiesterases was positively regulated by the VirRS-VR cascade (Table 1). The CpdC protein is similar to other 2′,3′-cyclic nucleotide 2′-phosphodiesterases from various bacteria. The putative product of *cpdC* had cell-wall sorting motifs (LPXTG) at their C-terminal ends [7] that might be required for sortase-mediated anchoring in the cell wall. It is believed that 2′,3′-cyclic nucleotide 2′-phosphodiesterase and its related nucleotidases play important roles in the recovery of inorganic phosphate from 3′ phosphonucleotides produced by the action of extracellular RNase. These enzymes are also important for the acquisition of carbon sources and energy by hydrolyzing non-transformable intermediates during the hydrolysis of RNA with RNase I [32].

Other genes encoding degradative enzymes are also affected by the system such as *N*-acetylglucosaminidases (CPE0289, CPE0818, and CPE0866).

### 3.6. Transporters

In a previous genome analysis, it was hypothesized that *C. perfringens* needs to import various nutrients (amino acids, sugars, minerals, etc.) to survive and grow in the host tissue, since it lacks many genes for amino acid biosynthesis [29]. In microarray experiments, the expression of several transporter genes (CPE0373, CPE0769, CPE1240, CPE1604, CPE1627-1630, CPE2345 and CPE2496) and phosphotransfer system (PTS) genes appeared to be affected by the VirRS-VR system (Table 1). The putative PTS-related systems (CPE0196-0199, CPE0317-0327, and CPE1463-1466) were positively regulated by the VirRS-VR cascade. The data indicate that the VirRS-VR system may also control part of the uptake and/or export of nutrients in *C. perfringens*.

### 3.7. Genes for energy production

The transcription of various genes for energy production was also affected by the VirRS-VR system. The VirRS-VR system positively influenced the expression of genes encoding electron transfer proteins (CPE0135 and CPE1014). Transcription of the genes required for carbohydrate metabolism also appeared to be affected by the VirRS-VR system. These genes were involved in the metabolism of

Please cite this article in press as: Ohtani K, et al., Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*, Anaerobe (2009), doi:10.1016/j.anaerobe.2009.10.003

**Table 1**
Genes that are affected by the VirR/VirS-VR-RNA cascade (identified by qRT-PCR).

| CPE#[a] | Strand | Gene | Product | mRNA amount in TS140 relative to Wild Type[b] | | | mRNA amount in TS140/pSB1036 relative to Wild Type[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1.5 h | 2 h | 2.5 h | 1.5 h | 2 h | 2.5 h |
| **CPE0036** | + | *plc* | phospholipase C | 0.36 ± 0.05 | 0.14 ± 0.03 | 0.18 ± 0.04 | 3 ± 0.27 | 2.99 ± 0.36 | 3.31 ± 0.34 |
| **CPE0079** | + | *fus* | elongation factor G | 0.3 ± 0.05 | 0.32 ± 0.03 | 1.41 ± 0.14 | 2.33 ± 0.4 | 2.93 ± 0.55 | 0.76[d] ± 0.04 |
| **CPE0085** | + | *mdh* | alcohol dehydrogenase | 0.48 ± 0.05 | 0.37 ± 0.05 | 0.42 ± 0.04 | 1.28 ± 0.13 | 1.62 ± 0.16 | 6.3 ± 0.97 |
| CPE0086 | + | *alf1* | fructose-bisphosphate aldolase | | | | | | |
| CPE0087 | + | *iolC* | myo-inositol catabolism protein | | | | | | |
| CPE0088 | + | *iolB* | myo-inositol catabolism protein | | | | | | |
| CPE0089 | + | *iolD* | myo-inositol catabolism protein | | | | | | |
| CPE0090 | + | - | dehydrogenase | | | | | | |
| CPE0091 | + | *iolE* | myo-inositol catabolism protein | | | | | | |
| CPE0092 | + | - | putative symporter YidK | 0.41 ± 0.07 | 0.45 ± 0.07 | 0.39 ± 0.05 | 1.35 ± 0.07 | 1.5 ± 0.06 | 5.74 ± 0.46 |
| CPE0093 | + | - | dehydrogenase | | | | | | |
| CPE0094 | + | - | hypothetical protein | | | | | | |
| CPE0095 | + | - | crotonase | | | | | | |
| CPE0096 | + | - | propionate CoA-transferase | | | | | | |
| CPE0097 | + | *acdS* | acyl-CoA dehydrogenase | | | | | | |
| **CPE0102** | + | - | hypothetical protein | 0.55 ± 0.05 | 0.28 ± 0.07 | 0.28 ± 0.05 | 2.89 ± 0.35 | 5.42 ± 0.96 | 5.58 ± 0.68 |
| CPE0115 | - | - | N-acetylmuramoyl-L-alanine amidase | | | | | | |
| CPE0116 | - | - | hypothetical protein | | | | | | |
| **CPE0117** | - | - | hypothetical protein | 2.78 ± 0.3 | 0.8 ± 0.09 | 1.06 ± 0.13 | 0.55 ± 0.09 | 1.3 ± 0.17 | 1.19 ± 0.15 |
| **CPE0135** | + | *rubY* | rubrerythrin | 0.52 ± 0.07 | 0.29 ± 0.06 | 0.41 ± 0.06 | 1.38 ± 0.07 | 3.15 ± 0.39 | 5.33 ± 0.56 |
| **CPE0168** | + | *arcA* | arginine deiminase | 0.47 ± 0.06 | 0.08 ± 0.04 | 0.07 ± 0.03 | 2.03 ± 0.19 | 1.66 ± 0.51 | 5.74 ± 0.46 |
| CPE0169 | + | *arcB* | ornithine carbamoyltransferase | | | | | | |
| CPE0170 | + | *arcD* | arginine/ornithine antiporter | | | | | | |
| CPE0171 | + | *arcC* | carbamate kinase | | | | | | |
| CPE0172 | + | *argR* | arginine repressor | | | | | | |
| **CPE0173** | + | *colA* | collagenase | 0.19 ± 0.04 | 0.09 ± 0.02 | 0.11 ± 0.03 | 4.35 ± 0.39 | 4.89 ± 0.29 | 6.63 ± 0.62 |
| **CPE0184** | + | *nanE* | N-acetylmannosamine-6-phosphate 2-epimerase | 0.2 ± 0.04 | 0.78 ± 0.1 | 0.77 ± 0.12 | 2.36 ± 0.45 | 2.81 ± 0.35 | 4.35 ± 0.47 |
| CPE0185 | + | *nanA* | N-acetylneuraminate lyase | | | | | | |
| CPE0186 | + | - | hypothetical protein | | | | | | |
| CPE0187 | + | - | hypothetical protein | | | | | | |
| CPE0188 | + | - | ROK family protein | | | | | | |
| CPE0189 | + | - | RpiR family transcriptional regulator | | | | | | |
| **CPE0196** | + | *ptiB* | PTS arbutin-like enzyme IIBC component | 0.18 ± 0.03 | 1.08 ± 0.14 | 2.22 ± 0.29 | 2.29 ± 0.16 | 0.86 ± 0.08 | 0.67 ± 0.08 |
| CPE0197 | + | - | hypothetical protein | | | | | | |
| CPE0198 | + | - | hypothetical protein | | | | | | |
| CPE0199 | + | *malH* | maltose-6'-phosphate glucosidase | | | | | | |
| **CPE0201** | - | - | 5'-nucleotidase, lipoprotein e(P4) family | 0.6 ± 0.05 | 0.46 ± 0.08 | 0.84 ± 0.08 | 1.27 ± 0.14 | 1.59 ± 0.08 | 3.81 ± 0.38 |
| **CPE0238** | + | - | hypothetical protein | 0.75 ± 0.08 | 0.47 ± 0.07 | 1.07 ± 0.22 | 1.32 ± 0.1 | 2.24 ± 0.28 | 5.69 ± 0.61 |
| **CPE0289** | - | - | endo-beta-N-acetylglucosaminidase | 0.17 ± 0.04 | 0.36 ± 0.08 | 1.11 ± 0.11 | 3.21 ± 0.35 | 2.37 ± 0.39 | 1.12 ± 0.14 |
| **CPE0317** | + | *fucK* | rhamnulokinase | 0.41 ± 0.07 | 0.08 ± 0.03 | 0.82 ± 0.06 | 2.32 ± 0.2 | 3.29 ± 0.29 | 1.34 ± 0.12 |
| CPE0318 | + | *fucI* | L-fucose isomerase | | | | | | |
| CPE0319 | + | *fucA* | L-fuculose phosphate aldolase | | | | | | |
| CPE0320 | + | - | hypothetical protein | | | | | | |
| CPE0321 | + | - | PTS system protein | | | | | | |
| CPE0323 | + | - | PTS system protein | 0.38 ± 0.09 | 0.1 ± 0.03 | 0.75 ± 0.08 | 1.97 ± 0.11 | 3.45 ± 0.26 | 1.29 ± 0.16 |
| CPE0324 | + | - | glycosyl hydrolase | | | | | | |
| CPE0326 | + | *lacA* | galactose-6-phosphate isomerase subunit LacA | | | | | | |
| CPE0327 | + | *lacB* | galactose-6-phosphate isomerase subunit LacB | | | | | | |
| **CPE0373** | + | - | suger ABC transporter | 0.78 ± 0.07 | 0.25 ± 0.07 | 0.9 ± 0.1 | 1.31 ± 0.11 | 2.27 ± 0.39 | 1.19 ± 0.08 |
| CPE0374 | + | *aga* | alpha-galactosidase | | | | | | |
| CPE0375 | + | - | endo-beta-galactosidase C | | | | | | |

Please cite this article in press as: Ohtani K, et al., Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*, Anaerobe (2009), doi:10.1016/j.anaerobe.2009.10.003

**Table 1 (*continued*)**

| CPE#[a] | Strand | Gene | Product | mRNA amount in TS140 relative to Wild Type[b] | | | mRNA amount in TS140/pSB1036 relative to Wild Type[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1.5 h | 2 h | 2.5 h | 1.5 h | 2 h | 2.5 h |
| **CPE0426** | + | - | hypothetical protein | 0.37 ± 0.09 | 0.34 ± 0.05 | 0.93 ± 0.06 | 1.62 ± 0.14 | 2.58 ± 0.28 | 1.02 ± 0.13 |
| **CPE0474** | + | *cysE* | serine O-acetyltransferase | 2.48 ± 0.23 | 2.65 ± 0.19 | 4.13 ± 0.19 | 0.7 ± 0.07 | 1.12 ± 0.1 | 0.76 ± 0.1 |
| CPE0475 | + | - | capsular polysaccharide biosynthesis protein | | | | | | |
| CPE0476 | + | - | capsular polysaccharide biosynthesis protein | | | | | | |
| CPE0477 | + | - | beta-glycosyltransferase | | | | | | |
| CPE0478 | + | - | capsular polysaccharide biosynthesis protein | | | | | | |
| CPE0479 | + | - | capsular polysaccharide biosynthesis protein | | | | | | |
| CPE0480 | + | - | capsular polysaccharide biosynthsis protein | | | | | | |
| CPE0481 | + | - | beta-1,4-galactosyltransferase | 2.23 ± 0.13 | 2.73 ± 0.21 | 3.79 ± 0.11 | 0.61 ± 0.05 | 1.32 ± 0.08 | 0.83 ± 0.09 |
| CPE0482 | + | - | glycero-phosphotransferase | | | | | | |
| CPE0483 | + | - | glycerol-3-phosphate cytidyltransferase | | | | | | |
| CPE0484 | + | - | capsular polysaccharide biosynthsis protein | | | | | | |
| CPE0486 | + | - | galactosyl transferase | | | | | | |
| CPE0487 | + | - | hypothetical protein | | | | | | |
| CPE0488 | + | - | capsular polysaccharide biosynthsis protein | | | | | | |
| CPE0490 | + | - | hypothetical protein | | | | | | |
| **CPE0491** | + | - | capsular polysaccharide biosynthsis protein | 2.68 ± 0.25 | 2.87 ± 0.25 | 0.57 ± 0.07 | 0.63 ± 0.07 | 0.9 ± 0.06 | 1.76 ± 0.11 |
| CPE0493 | + | - | capsular polysaccharide biosynthsis protein | | | | | | |
| CPE0494 | + | - | NDP-suger dehydrogenase | | | | | | |
| CPE0495 | + | - | mannose-1-phosphate guanylyltransferase | | | | | | |
| CPE0496 | + | - | N-acetyl-mannosamine transferase | | | | | | |
| CPE0497 | + | - | hypothetical protein | | | | | | |
| **CPE0500** | + | - | hexosyltransferase | 2.68 ± 0.35 | 2.06 ± 0.17 | 0.86 ± 0.08 | 0.36 ± 0.08 | 0.59 ± 0.03 | 1.13 ± 0.09 |
| CPE0501 | + | - | capsular polysaccharide biosynthsis protein | | | | | | |
| CPE0502 | + | - | lipopolysaccharide biosynthesis protein | | | | | | |
| **CPE0553** | + | *nanJ* | exo-alpha-sialidase | 0.88 ± 0.07 | 0.18 ± 0.06 | 0.85 ± 0.11 | 1.46 ± 0.15 | 2.38 ± 0.18 | 1.07 ± 0.15 |
| **CPE0659** | + | - | hypothetical protein | 0.32 ± 0.05 | 0.79 ± 0.06 | 1.34 ± 0.07 | 2.23 ± 0.22 | 1.16 ± 0.14 | 0.85 ± 0.08 |
| **CPE0725** | + | *nanI* | exo-alpha-sialidase | 0.79 ± 0.06 | 0.27 ± 0.05 | 0.85 ± 0.06 | 1.29 ± 0.16 | 2.71 ± 0.29 | 1.29 ± 0.06 |
| CPE0726 | + | - | hypothetical protein | | | | | | |
| **CPE0769** | + | *gutA* | sugar transport protein | 0.1 ± 0.02 | 1.14 ± 0.17 | 2.54 ± 0.1 | 3.48 ± 0.28 | 1.28 ± 0.05 | 0.59 ± 0.16 |
| **CPE0771** | + | *bgaL* | beta-galactosidase | 0.22 ± 0.04 | 1.45 ± 0.18 | 2.25 ± 0.39 | 2.59 ± 0.27 | 1.11 ± 0.22 | 0.54 ± 0.1 |
| **CPE0818** | + | - | endo-beta-N-acetylglucosaminidase | 0.29 ± 0.05 | 1.13 ± 0.17 | 1.51 ± 0.15 | 2.2 ± 0.28 | 0.6 ± 0.16 | 0.92 ± 0.1 |
| **CPE0856** | + | - | alpha-mannosidase | 0.35 ± 0.09 | 0.57 ± 0.04 | 1.76 ± 0.08 | 2.4 ± 0.25 | 1.94 ± 0.08 | 0.73 ± 0.12 |
| **CPE0866** | + | - | alpha-N-acetylglucosaminidase family protein | 0.48 ± 0.06 | 0.38 ± 0.05 | 2.26 ± 0.18 | 1.53 ± 0.11 | 2.25 ± 0.23 | 1.01 ± 0.12 |
| **CPE0892** | + | - | NADPH-dependent butanol dehydrogenase | 0.2 ± 0.09 | 0.36 ± 0.09 | 2.14 ± 0.38 | 2.1 ± 0.34 | 2.35 ± 0.38 | 0.78 ± 0.12 |
| **CPE0897** | + | *eutA* | reactivating factor for ethanolamine ammonia lyase | 0.09 ± 0.03 | 0.62 ± 0.06 | 2.56 ± 0.29 | 3.25 ± 0.5 | 2.19 ± 0.39 | 1.19 ± 0.28 |
| CPE0898 | + | *eutB* | ethanolamine ammonia lyase heavy chain | | | | | | |
| CPE0899 | + | *eutC* | ethanolamine ammonia-lyase small subunit | | | | | | |
| CPE0900 | + | *eutL* | ethanolamine utilization protein | | | | | | |
| CPE0901 | + | *pduJ* | propanediol utilization protein | | | | | | |
| CPE0902 | + | *adhE* | alcohol dehydrogenase | | | | | | |
| CPE0903 | + | *pduJ* | propanediol utilization protein | 0.07 ± 0.02 | 0.65 ± 0.07 | 2.33 ± 0.17 | 3.08 ± 0.36 | 2.02 ± 0.24 | 1.33 ± 0.15 |
| CPE0905 | + | *pduL* | propanediol utilization protein | | | | | | |
| CPE0906 | + | - | hypothetical protein | | | | | | |
| CPE0909 | + | *eutH* | ethanolamine utilization protein | | | | | | |
| **CPE1014** | - | - | rubredoxin/flavodoxin/oxidoreductase | 0.65 ± 0.09 | 0.39 ± 0.03 | 2.04 ± 0.26 | 1.56 ± 0.1 | 2.47 ± 0.3 | 0.83 ± 0.08 |
| **CPE1050** | + | - | 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase | 0.18 ± 0.07 | 0.68 ± 0.17 | 1.81 ± 0.17 | 2.34 ± 0.32 | 2.17 ± 0.26 | 0.87 ± 0.06 |
| **CPE1146** | + | *citC* | citrate (pro-3S)-lyase ligase | 0.31 ± 0.05 | 0.47 ± 0.05 | 2.5 ± 0.3 | 2.46 ± 0.39 | 1.79 ± 0.17 | 0.7 ± 0.17 |
| CPE1147 | + | *citG* | citrate lyase subunit gamma | | | | | | |
| CPE1148 | + | *citB* | citrate lyase beta subunit | | | | | | |
| CPE1149 | + | *citA* | citrate lyase alpha subunit | | | | | | |
| CPE1150 | + | - | triphosphoribosyl-dephospho-CoA synthase | | | | | | |
| CPE1151 | + | - | malate oxidoreductase | | | | | | |
| CPE1152 | + | *citN* | citrate/sodium symporter | | | | | | |

*(continued on next page)*

Please cite this article in press as: Ohtani K, et al., Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*, Anaerobe (2009), doi:10.1016/j.anaerobe.2009.10.003

**Table 1 (*continued*)**

| CPE#[a] | Strand | Gene | Product | mRNA amount in TS140 relative to Wild Type[b] | | | mRNA amount in TS140/pSB1036 relative to Wild Type[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 1.5 h | 2 h | 2.5 h | 1.5 h | 2 h | 2.5 h |
| **CPE1165** | + | *thd* | threonine dehydratase, catabolic | 0.3 ± 0.07 | 0.72 ± 0.07 | 1.18 ± 0.1 | 2.48 ± 0.34 | 1.74 ± 0.09 | 0.86 ± 0.06 |
| **CPE1169** | + | - | hypothetical protein | 2.55 ± 0.4 | 2.06 ± 0.08 | 1.48 ± 0.07 | 0.63 ± 0.09 | 0.77 ± 0.08 | 0.49 ± 0.07 |
| **CPE1240** | - | - | magnesium transporter | 0.38 ± 0.09 | 1.02 ± 0.11 | 1.04 ± 0.08 | 1.97 ± 0.11 | 0.93 ± 0.09 | 0.77 ± 0.06 |
| **CPE1316** | - | - | two-component sensor histidine kinase | 0.46 ± 0.05 | 0.48 ± 0.07 | 1.55 ± 0.09 | 1.7 ± 0.07 | 1.5 ± 0.08 | 0.58 ± 0.08 |
| **CPE1350** | + | *alf2* | fructose-1,6-bisphosphate aldolase, class II | 1.2 ± 0.12 | 1.13 ± 0.08 | 2.21 ± 0.16 | 0.78 ± 0.1 | 0.46 ± 0.05 | 0.33 ± 0.05 |
| CPE1463 | - | - | PTS system protein | | | | | | |
| CPE1464 | - | - | PTS system protein | | | | | | |
| CPE1465 | - | - | PTS system protein | | | | | | |
| **CPE1466** | - | - | PTS system protein | 0.37 ± 0.07 | 0.17 ± 0.06 | 0.36 ± 0.08 | 2.11 ± 0.22 | 0.18 ± 0.05 | 1.04 ± 0.18 |
| **CPE1467** | + | *pfk* | 6-phosphofructokinase | 0.91 ± 0.08 | 0.18 ± 0.06 | 1.05 ± 0.08 | 1.76 ± 0.08 | 2.09 ± 0.23 | 0.7 ± 0.03 |
| **CPE1523** | - | *nagL* | hyaluronidase | 0.71 ± 0.1 | 0.29 ± 0.06 | 0.89 ± 0.08 | 1.08 ± 0.15 | 2.29 ± 0.17 | 1.46 ± 0.1 |
| **CPE1529** | + | - | hypothetical protein | 2.36 ± 0.21 | 1.29 ± 0.06 | 1.09 ± 0.08 | 0.78 ± 0.05 | 0.89 ± 0.17 | 1.06 ± 0.1 |
| **CPE1604** | - | - | transporter, major facilitator family | 0.51 ± 0.06 | 0.33 ± 0.06 | 0.61 ± 0.06 | 1.84 ± 0.16 | 4.91 ± 0.33 | 7.75 ± 0.97 |
| CPE1626 | - | - | transcriptional regulator | | | | | | |
| CPE1627 | - | *rbsB* | ribose ABC transporter | | | | | | |
| CPE1628 | - | - | ribose ABC transporter | | | | | | |
| CPE1629 | - | *rbsC* | ribose ABC transporter | | | | | | |
| **CPE1630** | - | *rbsA* | ribose ABC transporter | 0.19 ± 0.05 | 0.3 ± 0.05 | 0.57 ± 0.07 | 3.18 ± 0.24 | 2.17 ± 0.24 | 2.12 ± 0.28 |
| CPE1875 | - | - | hypothetical protein | | | | | | |
| **CPE1876** | - | - | hypothetical protein | 0.64 ± 0.09 | 0.19 ± 0.03 | 0.82 ± 0.03 | 1.72 ± 0.07 | 3.14 ± 0.27 | 1.32 ± 0.13 |
| CPE2058 | - | - | glutamate decarboxylase | | | | | | |
| CPE2059 | - | - | hypothetical protein | | | | | | |
| **CPE2060** | - | - | glutamate gamma-aminobutyrate antiporter | 0.34 ± 0.06 | 0.19 ± 0.05 | 0.21 ± 0.06 | 1.9 ± 0.06 | 5.28 ± 0.37 | 8.55 ± 1.17 |
| CPE2076 | - | - | alpha-glucosidase | | | | | | |
| CPE2077 | - | - | ROK family protein | | | | | | |
| **CPE2078** | - | - | hypothetical protein | 0.37 ± 0.07 | 0.1 ± 0.03 | 0.43 ± 0.04 | 2.28 ± 0.15 | 3.99 ± 0.36 | 2.31 ± 0.37 |
| **CPE2162** | - | *cpdC* | 2`,3`-cyclic-nucleotide 2`-phosphodiesterase | 0.69 ± 0.03 | 0.47 ± 0.06 | 1.1 ± 0.07 | 1.35 ± 0.07 | 1.83 ± 0.16 | 4.6 ± 0.44 |
| CPE2336 | - | *gde* | glycogen debranching protein | | | | | | |
| CPE2337 | - | *glgP* | glycogen phosphorylase | | | | | | |
| CPE2338 | - | *malQ* | 4-alpha-glucanotransferase | | | | | | |
| CPE2339 | - | - | alpha-glucosidase | | | | | | |
| CPE2340 | - | *malR* | LacI family transcriptional regulator | | | | | | |
| **CPE2341** | - | - | maltose ABC transporter | 0.48 ± 0.08 | 0.37 ± 0.03 | 1.91 ± 0.23 | 3.32 ± 0.21 | 3.45 ± 0.26 | 0.59 ± 0.1 |
| **CPE2345** | - | - | maltose ABC transportor | 1.09 ± 0.14 | 0.45 ± 0.07 | 0.9 ± 0.09 | 0.7 ± 0.08 | 1.18 ± 0.09 | 2.14 ± 0.11 |
| **CPE2494** | + | - | transcriptional regulator | 0.27 ± 0.06 | 0.28 ± 0.03 | 0.75 ± 0.08 | 2.21 ± 0.12 | 2.22 ± 0.24 | 1.26 ± 0.08 |
| CPE2495 | + | *punA* | purine nucleoside phosphorylase | | | | | | |
| CPE2496 | + | - | nucleoside transporter | | | | | | |
| CPE2549 | - | - | hypothetical protein | | | | | | |
| CPE2550 | - | - | oxidoreductase, pyridine nucleotide-disulphide family | | | | | | |
| CPE2551 | - | *glpA* | glycerol-3-phosphate dehydrogenase | | | | | | |
| CPE2552 | - | *glpK* | glycerol kinase | | | | | | |
| **CPE2553** | - | *glpP* | glycerol uptake operon antiterminator | 0.32 ± 0.06 | 0.21 ± 0.06 | 2.31 ± 0.18 | 3.22 ± 0.45 | 3.13 ± 0.16 | 0.68 ± 0.09 |

[a] Putative operons deduced from microarray data are boxed. The first gene of each operon is in bold-face letter.

[b] Data from three independent qRT-PCR experiments (mean ± standard deviation).

a variety of sugars such as fructose (CPE1350 and CPE1467), *myo*-inositol (CPE0085 to CPE0097), sialic acid (CPE0184 to CPE0185, see above), galactose (CPE0374 and CPE0771), mannose (CPE0856) and fucose (CPE0317-0327). Induction of the *myo*-inositol operon (15.6 kb) by extracellularly added *myo*-inositol was already reported to be positively affected by the VirR/VirS system [15].

The operon *arcABDC* (CPE0168-CPE0172) for arginine deiminase (ADI) pathway, was strongly controlled by the VirRS-VR cascade (Table 1). The ADI pathway plays an important role in anaerobic energy production when glucose is not available in the environment, converting arginine into ornithine, ammonia, and $CO_2$ [10]. The generation of ammonia also plays an important role in defense against acidification [9]. The control of the *myo*-inositol and *arcABDC* operons by VirRS-VR cascade may be important for the energy metabolism in environments where glucose is not easily available.

Please cite this article in press as: Ohtani K, et al., Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*, Anaerobe (2009), doi:10.1016/j.anaerobe.2009.10.003

Many other genes or operons involved in the energy metabolism are also affected by VirRS-VR cascade, such as ethanolamine utilization operon (CPE0897-0909), citrate metabolism operon (CPE1146-1152), glycogen metabolism operon (CPE2336-2341), and glycerol metabolism (CPE2549-2553).

### 3.8. Hypothetical genes

We found that the VirRS-VR system also controlled 24 hypothetical genes of unknown functions (Table 1). The functional analysis of these VirRS-VR-regulated hypothetical genes will be required for understanding the VirRS-VR regulon in more detail.

## 4. Conclusions

In *C. perfringens*, VirR/VirS and VR-RNA have been thought to be very important for its pathogenicity by controlling the production of various known virulence factors, including alpha-toxin, kappa-toxin, theta-toxin, and sialidase. In this study, our understanding of the role of the VirR/VirS-VR-RNA system in *C. perfringens* has been significantly deepened. This will lead to an understanding of the lifestyle of the anaerobic pathogen *C. perfringens*.

The VirRS-VR regulon includes a variety of genes whose functions are closely related to cell survival in the host, i.e., destroying host cells and tissues (by toxins), degrading macromolecules into small nutrients (by enzymes), importing nutrients into the cell (by transporters), and finally metabolizing the nutrients intracellularly to produce energy and synthesize essential molecules required for cell survival and multiplication. Furthermore, the VirRS-VR system appears mainly to control the genes at the early- to mid-exponential growth phases, which exactly corresponds to the period when the cells are growing rapidly. Our data clearly indicate that the VirRS-VR system is essential part of the global regulators of genes required for cell survival and multiplication in special environments (i.e., poor nutrition); for humans, this may turn out to be a lethal pathogen – a flesh-eater. *C. perfringens* has developed a specific global regulatory system that controls genes that are required for survival in unique environmental conditions, including the human body. Elucidation of the fine mechanism of regulation by the VirRS-VR system will lead to identification of suitable targets for effective prevention and/or treatment of the *C. perfringens* infection. Further molecular studies on the *C. perfringens* regulatory systems should be indispensable to this purpose.

## Acknowledgments

This work was supported by KAKENHI (Grant-in-Aid for Scientific Research) on Priority Areas "Applied Genomics" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by Health and Labor Sciences Research Grants (Research on Food Safety).

## Appendix. : Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.anaerobe.2009.10.003.

## References

[1] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
[2] Awad MM, Bryant AE, Stevens DL, Rood JI. Virulence studies on chromosomal α-toxin and θ-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of α-toxin in *Clostridium perfringens*-mediated gas gangrene. Mol Microbiol 1995;15:191–202.
[3] Ba-Thein W, Lyristis M, Ohtani K, Nisbet IT, Hayashi H, Rood JI, et al. The *virR/virS* locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. J Bacteriol 1996;178:2514–20.
[4] Banu S, Ohtani K, Yaguchi H, Swe T, Cole ST, Hayashi H, et al. Identification of novel VirR/VirS-regulated genes in *Clostridium perfringens*. Mol Microbiol 2000;35:854–64.
[5] Cheung JK, Dupuy B, Deveson DS, Rood JI. The spatial organization of the VirR boxes is critical for VirR-mediated expression of the perfringolysin O gene, *pfoA*, from *Clostridium perfringens*. J Bacteriol 2004;186:3321–30.
[6] Cheung JK, Rood JI. The VirR response regulator from *Clostridium perfringens* binds independently to two imperfect direct repeats located upstream of the *pfoA* promoter. J Bacteriol 2000;182:57–66.
[7] Comfort D, Clubb RT. A comparative genome analysis identifies distinct sorting pathways in gram-positive bacteria. Infect Immun 2004;72:2710–22.
[8] Corfield AP, Wagner SA, Clamp JR, Kriaris MS, Hoskins LC. Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. Infect Immun 1992;60:3971–8.
[9] Cotter PD, Hill C. Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol Mol Biol Rev 2003;67:429–53.
[10] Cunin R, Glansdorff N, Pierard A, Stalon V. Biosynthesis and metabolism of arginine in bacteria. Microbiol Rev 1986;50:314–52.
[11] DeRisi JL, Iyer VR, Brown PO. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 1997;278:680–6.
[12] Flores-Diaz M, Alape-Giron A, Clark G, Catimel B, Hirabayashi Y, Nice E, et al. A cellular deficiency of gangliosides causes hypersensitivity to *Clostridium perfringens* phospholipase C. J Biol Chem 2005;280:26680–9.
[13] Hatheway CL. Toxigenic clostridia. Clin Microbiol Rev 1990;3:66–98.
[14] Hynes WL, Walton SL. Hyaluronidases of Gram-positive bacteria. FEMS Microbiol Lett 2000;183:201–7.
[15] Kawsar HI, Ohtani K, Okumura K, Hayashi H, Shimizu T. Organization and transcriptional regulation of *myo*-inositol operon in *Clostridium perfringens*. FEMS Microbiol Lett 2004;235:289–95.
[16] Lyristis M, Bryant AE, Sloan J, Awad MM, Nisbet IT, Stevens DL, et al. Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. Mol Microbiol 1994;12:761–77.
[17] Mahony DE, Moore TJ. Stable L-forms of *Clostridium perfringens* and their growth on glass surfaces. Can J Microbiol 1976;22:953–9.
[18] Matsushita O, Okabe A. Clostridial hydrolytic enzymes degrading extracellular components. Toxicon 2001;39:1769–80.
[19] Myers GS, Rasko DA, Cheung JK, Ravel J, Seshadri R, DeBoy RT, et al. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. Genome Res 2006;16:1031–40.
[20] O'Brien DK, Melville SB. Multiple effects on *Clostridium perfringens* binding, uptake and trafficking to lysosomes by inhibitors of macrophage phagocytosis receptors. Microbiology 2003;149:1377–86.
[21] Ohtani K, Kawsar HI, Okumura K, Hayashi H, Shimizu T. The VirR/VirS regulatory cascade affects transcription of plasmid-encoded putative virulence genes in *Clostridium perfringens*. FEMS Microbiol Lett 2003;222:137–41.
[22] Ohtani K, Takamura H, Yaguchi H, Hayashi H, Shimizu T. Genetic analysis of the *ycgJ-metB-cysK-ygaG* operon negatively regulated by the VirR/VirS system in *Clostridium perfringens*. Microbiol Immunol 2000;44:525–8.
[23] Okumura K, Ohtani K, Hayashi H, Shimizu T. Characterization of genes regulated directly by the VirR/VirS system in *Clostridium perfringens*. J Bacteriol 2008;190:7719–27.
[24] Petit L, Gibert M, Popoff MR. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol 1999;7:104–10.
[25] Rood JI. Virulence genes of *Clostridium perfringens*. Annu Rev Microbiol 1998;52:333–60.
[26] Rossolini GM, Schippa S, Riccio ML, Berlutti F, Macaskie LE, Thaller MC. Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. Cell Mol Life Sci 1998;54:833–50.
[27] Sakaki K, Tashiro K, Kuhara S, Mihara K. Response of genes associated with mitochondrial function to mild heat stress in yeast *Saccharomyces cerevisiae*. J Biochem 2003;134:373–84.
[28] 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 the perfringolysin O, collagenase, and hemagglutinin in *Clostridium perfringens*. J Bacteriol 1994;176:1616–23.
[29] Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, et al. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc Natl Acad Sci U S A 2002;99:996–1001.
[30] Shimizu T, Shima K, Yoshino K, Yonezawa K, Shimizu T, Hayashi H. Proteome and transcriptome analysis of the virulence genes regulated by the VirR/VirS system in *Clostridium perfringens*. J Bacteriol 2002;184:2587–94.
[31] 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 2002;43:257–65.
[32] Trulzsch K, Roggenkamp A, Pelludat C, Rakin A, Jacobi C, Heesemann J. Cloning and characterization of the gene encoding periplasmic 2′,3′-cyclic phosphodiesterase of *Yersinia enterocolitica* O:8. Microbiology 2001;147:203–13.
[33] Walters DM, Stirewalt VL, Melville SB. Cloning, sequence, and transcriptional regulation of the operon encoding a putative *N*-acetylmannosamine-6-phosphate epimerase (*nanE*) and sialic acid lyase (*nanA*) in *Clostridium perfringens*. J Bacteriol 1999;181:4526–32.

Please cite this article in press as: Ohtani K, et al., Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*, Anaerobe (2009), doi:10.1016/j.anaerobe.2009.10.003

The Journal of Microbiology (2010) Vol. 48, No. 1, pp. 96-101
DOI 10.1007/s12275-009-0384-3


# Transcriptional Regulation of *hemO* Encoding Heme Oxygenase in *Clostridium perfringens*

**Sufi Hassan, Kaori Ohtani, Ruoyu Wang, Yonghui Yuan, Yun Wang, Yumi Yamaguchi, and Tohru Shimizu***

*Department of Bacteriology, Graduate School of Medical Science, Kanazawa University, Kanazawa 920-8640, Japan*

(Received December 11, 2009 / Accepted December 28, 2009)

**A Gram-positive anaerobic pathogen, *Clostridium perfringens*, causes clostridial myonecrosis or gas gangrene in humans by producing numerous extracellular toxins and enzymes that act in concert to degrade host tissues. The ability of infectious bacteria to acquire sufficient iron during infection is essential for the pathogen to cause disease. In the *C. perfringens* strain 13 genome, a heme oxygenase gene homologue (CPE0214, *hemO*) was found and its role was examined. The purified recombinant HemO protein showed heme oxygenase activity that can convert heme to biliverdin. *hemO* transcription was induced in response to extracellular hemin in a dose-dependent manner. The global two-component VirR/VirS regulatory system and its secondary regulator VR-RNA had positive regulatory effects on the transcription of *hemO*. These data indicate that heme oxygenase may play important roles in iron acquisition and cellular metabolism, and that the VirR/VirS-VR-RNA system is also involved in the regulation of cellular iron homeostasis, which might be important for the survival of *C. perfringens* in a human host.**

*Keywords*: *C. perfringens*, heme oxygenase, two-component system, genetic regulation

The Gram-positive anaerobic pathogen *Clostridium perfringens* is a causative agent of clostridial myonecrosis (gas gangrene) and mild diarrhea in humans (McDonel, 1980; Hatheway, 1990). The organism produces numerous toxins and enzymes that act in concert to degrade various components of human tissues, resulting in severe myonecrosis (Rood, 1998; Petit *et al.*, 1999). In *C. perfringens*, genes for many toxins and enzymes are regulated by the two-component VirR/VirS system and its secondary regulator, VR-RNA (Lyristis *et al.*, 1994; Shimizu *et al.*, 1994, 2002). Recent microarray analysis suggested that 147 genes (30 single genes and 21 putative operons) are regulated by the VirR/VirS-VR-RNA regulatory cascade (Ohtani *et al.*, 2009). Many genes for putative virulence factors, transporters, and metabolic enzymes are included in the regulon, suggesting that the VirR/VirS-VR-RNA regulatory system controls multiple cellular functions to survive and multiply in the host (Ohtani *et al.*, 2009).

Generally, iron is an essential nutrient required for the survival of most bacteria. The ability of pathogenic bacteria to acquire sufficient iron during infection is essential for pathogens to cause disease. The levels of extracellular iron available within the host are limited (Wilks and Schmitt, 1998), since much of the extracellular iron in eukaryotes is sequestered by the iron-binding proteins transferrin and lactoferrin, while intracellular iron is commonly bound to heme, which is the most abundant source of iron in humans (Schmitt, 1997). Many pathogenic bacteria possess specific heme uptake systems that harness heme iron for metabolic needs. Externally supplied heme cannot satisfy the cellular requirement for iron without the involvement of heme degradation (Zhu *et al.*, 2000). Heme oxygenase, an enzyme that removes iron from the heme moiety, has been identified in a few pathogenic bacterial species, including *Corynebacterium diphtheriae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. In these organisms, heme oxygenase is required for the use of heme as a source of iron (Wyckoff *et al.*, 2004). By genome analysis of *C. perfringens* strain 13, we found that *C. perfringens* possesses a homolog for heme oxygenase (CPE0214) in the chromosome (Shimizu *et al.*, 2002). Investigation of the physiological role of heme oxygenase would be very important for understanding the pathogenicity of *C. perfringens*.

In this study, we determined that CPE0214 (*hemO*) encodes heme oxygenase in *C. perfringens*. A heme catalytic assay using purified recombinant HemO protein indicated that HemO has catalytic activity and can convert hemin to biliverdin. Northern analysis also indicated that the *hemO* gene was positively regulated at the transcriptional level by the VirR/VirS-VR-RNA regulatory cascade.

## Materials and Methods

### Strains, media, plasmids, and culture conditions

*C. perfringens* strain 13 and its derivatives, TS133 (*virR*$^-$) and TS140 (*vrr*$^-$), as well as their complemented strains TS133 with pTS405 (plasmid containing intact *virR/virS*) and TS140 with pSB1031 (plasmid carrying intact *vrr*) (Shimizu *et al.*, 1994, 2002), were cultured in GAM (Gifu anaerobic medium; Nissui, Japan) at 37°C under anaerobic conditions as described previously (Shimizu *et al.*, 1994). *Escherichia coli* strain DH5α was cultured under standard conditions (Sambrook *et al.*, 1989). *E. coli* strain BL21 (Studier and Moffatt, 1986) was used as a host cell for pGEX-3X and cultured in Luria

* For correspondence. E-mail: tshimizu@med.kanazawa-u.ac.jp; Tel: +81-76-265-2200; Fax: +81-76-234-4230

Bertani (LB) or 2× YT (16 g tryptone, 10 g yeast extract, 5 g NaCl/L) medium. A chemically defined medium described by Riha and Solberg (1971) was also used to grow *C. perfringens* strain 13. Hemin (bovine) was obtained from Nacalai Tesque Inc. (Japan), and a solution containing 1 mM of hemin was prepared as described previously (Yoshida and Kikuchi, 1978). Erythromycin (50 μg/ml), chloramphenicol (25 μg/ml), and ampicillin (50 μg/ml) were added to each medium for cultures of appropriate bacterial strains.

### DNA manipulation

General recombinant DNA techniques were performed as described previously (Sambrook *et al.*, 1989) unless otherwise noted. *C. perfringens* strains were transformed by electroporation-mediated transformation as described previously (Shimizu *et al.*, 1994).

### Expression of the GST-CPE0214 fusion protein

To construct the plasmid for expressing the CPE0214-GST fusion protein, a PCR fragment amplified with primers CPE0214-BamHI (5′-AAGGATCCTGAACTCATTTATGATGGATAT-3′) and CPE0214-EcoRI-2 (5′-AAGAATTCATTGGGAGTAAGCACTATAG-3′) was digested with *Bam*HI and *Eco*RI, and then ligated to the *Bam*HI and *Eco*RI sites of pGEX-3X. The resulting plasmid (named pBE510) was transformed into *E. coli* BL21, which was cultured in 5 ml LB medium with 50 μg/ml ampicillin and 0.1% glucose for 12 h at 37°C. Fifty microliters of the overnight culture was inoculated into 5 ml LB medium and cultured under the same conditions. When $OD_{600}$ reached 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the culture was incubated for 8 h at 37°C. *E. coli* BL21 containing pGEX-3X was also cultured in the same manner and used as a control.

### Purification of the CPE0214 product

The GST-CPE0214 fusion protein was purified from the *E. coli* BL21-codon plus (DE3) (Stratagene, USA) transformed with pBE510. The strain was inoculated in 5 ml LB medium containing 50 μg/ml of ampicillin and cultured overnight with gentle agitation at 37°C. The primary culture was inoculated into 2× YT medium at a 1% concentration, which was then incubated for 2 h at 37°C. When $OD_{600}$ reached 0.5, IPTG was added to a final concentration of 1 mM, and the incubation was continued for 4 h. Cells were harvested by centrifugation at 7,000 rpm for 20 min, washed in phosphate-buffered saline (PBS, pH 7.4) three times, and lysed with 75 units of Benzonase (Merck, Germany) in 1 ml Bugbuster cell wall lysis buffer (Novagen, USA) with rotation for 1 h at 4°C. The lysed cell suspension was centrifuged at 7,000 rpm for 30 min, and the supernatant was collected. Then, 750 μl of glutathione-Sepharose was added and the mixture was incubated with rotation for 30 min at 4°C. After centrifugation, the Sepharose pellet was mixed again with 750 μl of glutathione-Sepharose and the mixture was incubated with rotation for 30 min at 4°C. After centrifugation, the supernatant was mixed again with 750 μl of glutathione-Sepharose, and the same procedure was done to collect a Sepharose pellet, which was added to the previously collected pellet. Six units of factor Xa enzyme in 2 ml Xa buffer were added and mixed with the precipitated Sepharose, and the rotation was continued at 4°C for 16 h. The mixture was centrifuged and the supernatant was collected as GST-removed CPE0214. The protein solution was concentrated using a Microcon YM 30 spin column (the MW cutoff was 30 kDa). Protein concentration was measured using a protein assay kit (Bio-Rad Laboratories, USA). The CPE0214 protein was then stored at -80°C until use.

### Reconstitution of HemO with hemin and measurement of HemO activity

The HemO (CPE0214)-heme complex was prepared as described previously (Zhu *et al.*, 2000; Zhang *et al.*, 2004). Briefly, hemin was added gradually to the purified HemO (10 μM) to give a final 2:1 heme:protein ratio in a total of 1 ml of 100 mM potassium phosphate buffer (pH 7.4). An equal volume of hemin was added to the potassium phosphate buffer (pH 7.4) without HemO, and this solution was used as a reference. Absorbance at 405 nm was measured with a BioWave II spectrophotometer. Then, ascorbic acid at a final concentration of 5 mM was added to the heme-HemO complex, and spectral changes between 300 and 750 nm were recorded at 10 and 40 min after the addition of ascorbic acid.

### Northern hybridization

Total RNA was extracted from *C. perfringens* according to a method described previously (Aiba *et al.*, 1981). A 499-bp DNA probe for *hemO* was obtained by PCR with CPE214F (5′-AAACATAAGAATTAGCAACT-3′) and CPE0214R (5′-AGCTTCTACTGAAAGCTACG-3′). Northern hybridization was performed as described previously (Kobayashi *et al.*, 1995; Ba-Thein *et al.*, 1996), with the exceptions that the DNA fragment specific for *hemO* was labeled with an AlkPhos-direct kit (Amersham Pharmacia Biotech, UK) and signals were detected by CDPstar chemiluminescence.

## Results and Discussion

### Similarity of the CPE0214 to other clostridial heme oxygenases

The product of CPE0214 of *C. perfringens* strain 13, originally annotated for heme oxygenase (Shimizu *et al.*, 2002), was compared with other clostridial proteins using a BLAST program. The amino acid sequence of CPE0214 was highly similar to those of heme oxygenases found in other *C. perfringens* strains (ATCC 13124 & F4969), with 98% identity, as well as heme oxygenases in *C. tetani* (strain E88) and *C. novyi* (strain NT), with 62% and 53% identity, respectively. Their amino acid sequence alignment using CLUSTAL W (http://www.genome.jp/en/) is shown in Fig. 1. The amino acid sequence alignment indicated that CPE0214 showed a significant number of identical amino acid residues between other clostridial heme oxygenases, as well as those from other various species (more detailed information is available at http://pfam.sanger.ac.uk/family/PF01126). Maximum sequence similarity was observed within the 116 to 136 amino acid region (ELLvAHAYTRYLADLFGGRTI) in CPE0214 of *C. perfringens* strain 13 (Fig. 1), indicating that this region is highly conserved among all clostridial heme oxygenases and that CPE0214 may encode a functional heme oxygenase in *C. perfringens* strain 13.

### Expression of the GST-CPE0214 fusion protein in *E. coli*

To characterize the activity of the CPE0214 product, we made a GST-CPE0214 fusion protein. *E. coli* BL21 carrying pBE510 (pGEX-3X with a CPE0214-coding region inserted) was cultured in LB medium, and the expression of the GST-CPE0214 fusion protein was induced by adding IPTG. At 8 h after IPTG induction, the culture medium of *E. coli* BL21 (pBE510) turned pale green, while no color change was observed in the culture of *E. coli* BL21 carrying the empty

```
C. perfringens str. 13       ----------MNSFMMDIKNNSNDLHAVAEKTGFLKRLLEGKASTESYAEYLYNLY  46
C. perfringens ATCC13124     ----------MNSFMMDIKNNSNDLHAVAEKTGFLKRLLEGKASTESYAEYLYNLY  46
C. perfringens F4969         ----------MNSFMMDIKNNSNDLHAVAEKTGFLKRLLEGKASTESYAEYLYNLY  46
C. tetani E88                MSIINNYIKEVFVMENTFLNEIRLNSSKLHDMAEHTGFIKRLIEGNANVTTYAEYIYNLY  60
C. novyi NT                  ----------MINEFMKKIRFESESLHDMAEHTGFINRLIEGNASKETYGKYIYNLY  47
                                        * *: .*: :*..** :**:***::**:**:*.  :*.:*:****

C. perfringens str. 13       EVYNAIEVNLEKCKDNKVVKDFVLPEIYRAEAILKDLKFLLEENLNTMKPLASTRAYVAR 106
C. perfringens ATCC13124     EVYNAIEVNLEKCKDNKVVKDFVLPEIYRAEAILKDLKFLLGENLNTMKPLASTRAYVAR 106
C. perfringens F4969         EVYNSIEVNLEKCKDNKVVKDFVLPEIYRAEAILKDLKFLLGENLNTMKPLASTRAYVAR 106
C. tetani E88                HIYNAIESNLEKNKGNKYIKDFALPEVYRAEAIMKDVKYLLKDKLDSMEPLISTKVFVNR 120
C. novyi NT                  HVYKAIEDNLEKNKSNENVANFALPDVYRSEEISKDVKSILGEDYEKVPLLMSTKVFVNR 107
                             .:*::** **** *.*: : :*.**::**:* * **:* :* :. :.:  * **:.:* *

C. perfringens str. 13       INEIGETAPELLVAHAYTRYLADLFGGRTIYGMVKDLYKIDEEGLNYYKYETLSDGSEMK 166
C. perfringens ATCC13124     INEIGETAPELLVAHAYTRYLADLFGGRTIYGMVKDLYKIDEEGLNYYKYETLSDGPEMK 166
C. perfringens F4969         INEIGETAPELLVAHAYTRYLADLFGGRTIYGMVKDLYKIDEEGLNYYKYETLSDGPEMK 166
C. tetani E88                INHIGEKNKELLIAHAYTRYLADLFGGRTIYQIVKENYKIDDKGLNYYIFHEIND---LK 177
C. novyi NT                  INFIGNSDPELLIAHAYTRYLADLFGGRTILEIIKKHYKLEDESLNYYVFPQIKD---FR 164
                             ** **:.  ***:****************  ::*. **:::::.**** :  :.*   ::

C. perfringens str. 13       GFVMNYHNKLNNIELNEEMKERFINEVANSYVYNIAISNELAFIRFNR- 204
C. perfringens ATCC13124     GFVMNYHNKLNNIELNEEMKERFINEVANSYVYNIAISNELDFIRFNR- 204
C. perfringens F4969         GFVMNYHNKLNNIELNEEMKERFINEVANSYVYNIAISNELDFIRFNR- 204
C. tetani E88                NFVMGYHEKLNNIKFDETLKKDFINEISISYIYNISISNELEFDRFK- 224
C. novyi NT                  QFVMQYHGKLNALNLSESMQEKFLNEISISYIYNISISNELEFLEYHKK 213
                             *** ** *** :::.* ::: *:**:: **:***:***** * .::
```

**Fig. 1.** Amino acid sequence alignment of heme oxygenase proteins from Clostridial species; *C. perfringens* strain 13 (NP_561130.1), *C. perfringens* ATCC 13124 (YP_694668.1|), *C. perfringens* F4969 (ZP_02639684.1), *C. tetani* ED88 (NP_783005.1), and *C. novyi* NT (YP_878917.1). Identical and similar amino acids are shown with asterisks and dots, respectively. The identical amino acid region is boxed.

pGEX-3X vector (Fig. 2A). The coloration was probably due to the formation of biliverdin, which suggests that the heme oxygenase activity of the GST-CPE0214 protein converted the endogenous heme of *E. coli* into biliverdin just as reported previously (Bruggemann *et al.*, 2004). From these results, we may assume that the product of CPE0214 encodes heme oxygenase (HemO) of *C. perfringens*.

To purify the recombinant HemO protein from *E. coli*, we used *E. coli* BL21-codon plus (DE3) as a host cell to increase the amount of fusion protein. After cell lysis, an approximately 50-kDa protein was clearly detected by SDS-PAGE. It corresponded well to the estimated size (50.7 kDa) of the GST-HemO fusion protein (Fig. 2B; lane 3). The expressed fusion protein was purified from the crude extract of *E. coli* cells and digested with factor Xa to remove the GST moiety (Fig. 2B; lane 4). As indicated by SDS-PAGE, the purified HemO showed a single band at approximately 24.7 kDa, which was expected from the HemO amino acid composition. Therefore, this purified 24.7-kDa HemO protein was used in the subsequent experiments.

## Heme oxygenase activity of HemO

The purified HemO protein was further tested for its heme oxygenase activity. The purified HemO was mixed with hemin to make a heme-HemO complex. When this complex was monitored photometrically, its maximum absorbance was detected at 405 nm (Soret band). This ferric ($Fe^{3+}$) heme-HemO complex was reacted in the presence of ascorbic acid, and its absorbance (from 300 nm to 750 nm) was scanned by a spectrometer (Fig. 3). The Soret band gradually decreased and shifted from 405 nm to 412 nm (Fig. 3), indicating that a ferrous dioxygen complex ($Fe^{2+}$ oxy-heme:HemO) was formed. Within 40 min, the ferrous dioxygen complex was first converted to a ferric biliverdin-HemO complex (represented by the decrease in the Soret band), and then the ferric biliverdin-HemO complex was degraded into free biliverdin (represented by the formation of a broad peak around 680 nm) (Fig. 3).

The previously characterized heme oxygenase (HemT) of *C. tateni* showed similar characteristics in terms of its heme cleavage reaction (Bruggemann *et al.*, 2004). First, the enzyme formed a 1:1 complex with hemin, resulting in a maximum absorbance of the hemin-HemT complex at 405 nm. To demonstrate the heme oxygenase catalytic activity, ascorbic acid was used as an electron donor in the oxidative degradation of hemin by HemT. The addition of ascorbic acid to the hemin-HemT complex initiated heme degradation. In a period of 2 h, the Soret band at 405 nm disappeared and a broad absorption peak appeared around 680 nm, indicating that the hemin was converted to biliverdin.

Since the same heme-degrading reaction was observed in HemO, we concluded that HemO of *C. perfringens* also has heme oxygenase activity that generates free iron from heme, resulting in the formation of biliverdin. The heme oxygenase of *C. tetani* was proposed to play an important role in iron metabolism at the site of infection in the human body

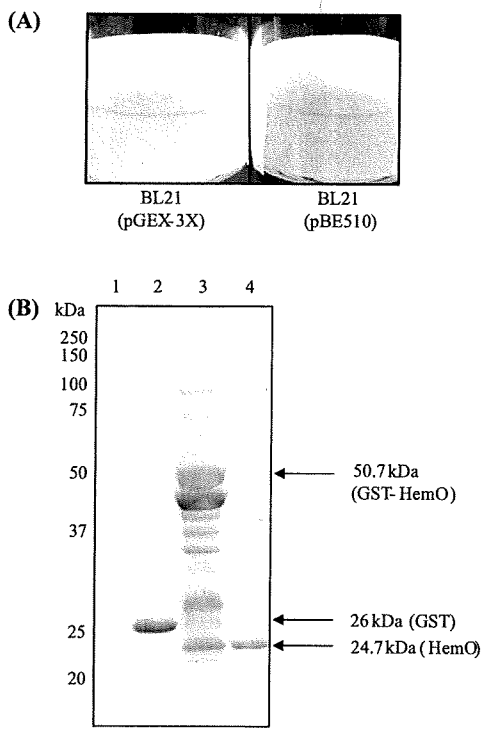


**Fig. 2.** Expression and purification of the CPE0214 (HemO) protein. (A) *E. coli* BL21 carrying pGEX-3X (control) and pBE510 (pGEX-3X+*hemO*) were cultured in LB with 1 mM IPTG for 8 h. Note that the medium of BL21 (pBE510) turned a pale green color. (B) Purification of HemO. Lanes: 1, protein size marker; 2, GST purified from *E. coli* BL21-codon plus carrying pGEX-3X; 3, GST-HemO fusion protein purified from *E. coli* BL21-codon plus carrying pBE510; 4, HemO protein purified from the GST-HemO fusion protein by cleavage with factor Xa.

(Bruggemann *et al.*, 2004). Moreover, heme oxygenase is a good candidate for involvement in enhanced aerotolerance in the wound environment, since heme oxygenase could be an oxygen scavenger producing highly effective antioxidants, including biliverdin. Therefore, it might be reasonable to think that the same anaerobic wound-infecting pathogen, *C. perfringens*, also uses heme oxygenase to establish an anoxic environment, enabling the organism to survive at the site of infection.

## Transcription analysis of *hemO*

The transcription profile of *hemO* in *C. perfringens* strain 13 was examined by Northern hybridization analysis. Total RNA was prepared from the strain at different culture stages (1 h to 5 h from the start of culture), and Northern hybridization was performed by using a PCR-amplified *hemO* DNA probe. As a result, approximately 1.0-kb mRNA was clearly detected. This size corresponded well with the length of the *hemO* gene (645 bp). The *hemO* mRNA appeared from the early log to the stationary growth phase. The mRNA amount was highest at 2 h and 3 h of culture and decreased slightly in the later growth phases (Fig. 4A). The sustained transcription of *hemO* seemed

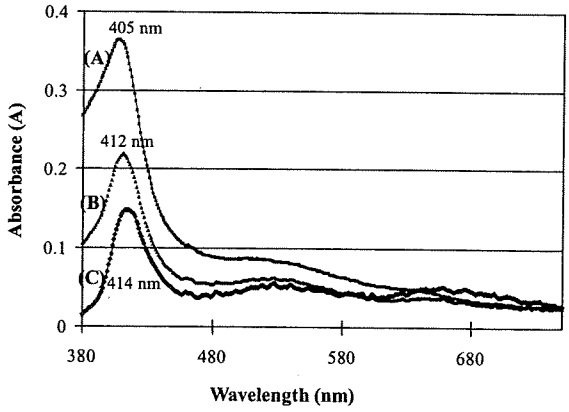


**Fig. 3.** Heme degradation to biliverdin catalyzed by HemO. Heme conversion was initiated by the addition of ascorbic acid (AA) to the HemO-heme complex, and spectral changes between 300 and 750 nm were recorded at 10 and 40 min. (A) HemO-heme complex (before addition of AA); (B) 10 min after AA addition; (C) 40 min after AA addition. Note the shifting of the Soret band from 405 nm to 414 nm (formation of ferrous dioxygen complex) and the appearance of a broad peak around 680 nm (biliverdin production).

to be unique among the gene transcriptions of *C. perfringens*, since the transcription of many *C. perfringens* genes has been reported to peak in the early-log phase and to quickly disappear by the late-log phase (Ba-Thein *et al.*, 1996; Okumura *et al.*, 2008). These findings may suggest that the prolonged transcription of *hemO* is due to an adaptive response required for the maintenance of metabolic activity related to iron acquisition.

## Induction of *hemO* transcription with hemin

Further Northern hybridization analysis was performed to examine hemin's effect on the transcription of *hemO*. Wild-type strain 13 was cultured in chemically defined medium for 10 h, and then 0.1 μM of hemin or 0.2 μM of iron ($FeSO_4 \cdot 7H_2O$) was added to the medium. Total RNA was isolated at 15 min after the addition of hemin or iron and was subjected to Northern analysis. Transcription of *hemO* was strongly induced by hemin but repressed by the addition of iron (Fig. 4B). Furthermore, the transcription of *hemO* increased as the concentration of extracellular hemin increased (Fig. 4C), indicating that the expression of *hemO* is actively induced when its substrate (hemin) exists in the environment in a dose-dependent manner. Conversely, when free iron is available, the *hemO* gene is not transcribed, suggesting some additional regulatory mechanism may exist to control iron homeostasis. Further studies are needed to elucidate the regulation of iron metabolism in *C. perfringens*.

## Regulation of *hemO* by the VirR/VirS-VR-RNA system

A recent study of the VirR/VirS-VR-RNA regulon using a *C. perfringens* microarray (Ohtani *et al.*, 2009) showed that 147 genes are regulated by the VirR/VirS-VR-RNA system. The *hemO* gene (CPE0214) was not included in the predicted regulon, since its expression level was slightly under the

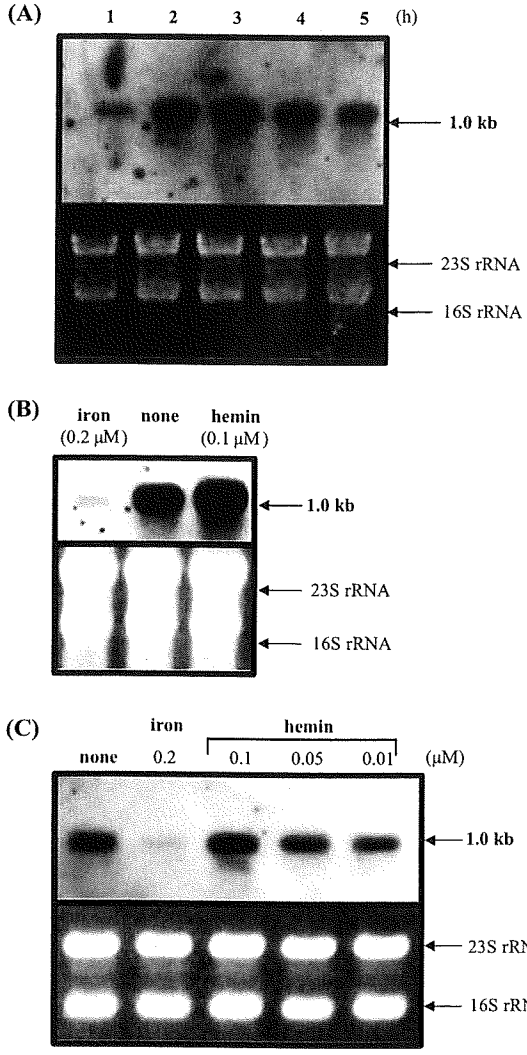


**Fig. 4.** Transcriptional analysis of *hemO* by Northern hybridization. (A) Growth-phase-dependent expression of the *hemO* mRNA (1 h to 5 h). The mRNA of *hemO* is indicated by an arrow along its length. (B) Transcriptional change of *hemO* in the presence of 0.2 μM iron ($FeSO_4 \cdot 7H_2O$) or 0.1 μM hemin. Total RNA was extracted from *C. perfringens* cells at 15 min after addition of iron or hemin and subjected to Northern hybridization. (C) Dose-dependent induction of *hemO* by hemin. The indicated concentrations of hemin (0.1, 0.05, and 0.01 μM) were added to the *C. perfringens* cultures, and total RNA was prepared from the cells at 15 min after the addition. Each lane was loaded with 10 μg of total RNA. Photographs of the EtBr-stained gel are shown with the locations of 23S and 16S rRNAs.

threshold set in the microarray analysis. To check the transcriptional regulation of *hemO* by the VirR/VirS and/or VR-RNA system, the transcription of *hem*O in *C. perfringens* strains 13(pJIR418) (wild type), TS133(pJIR418) (*virR*$^-$), TS133(pTS405) (*virR/virS*$^+$) (Okumura *et al.*, 2008), TS140 (pJIR418) (*vrr*$^-$), and TS140(pSB1031) (*vrr*$^+$) was examined by Northern hybridization analysis. At 2 h of culture, *hemO*

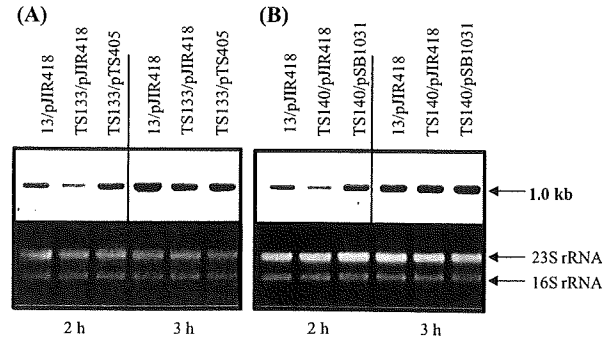


**Fig. 5.** Regulation of *hemO* by VirR/VirS and the VR-RNA system. Total RNA was extracted from the *C. perfringens* strains at 2 and 3 h from the start of the culture. (A) The wild-type strain 13(pJIR418), TS133(pJIR418)(*virR*$^-$), and TS133(pBT405) (*virR/virS*$^+$) were examined. (B) The wild-type strain 13(pJIR418), TS140(pJIR418)(*vrr*$^-$), and TS140(pSB1031) (*vrr*$^+$) were examined. Each lane was loaded with 10 μg of total RNA. Photographs of the EtBr-stained gel are shown with the locations of 23S and 16S rRNAs.

transcription decreased in TS133(pJIR418) and recovered in TS133(pTS405) (Fig. 5A). Similarly, it decreased in TS140 (pJIR418) at 2 h of culture compared with 13(pJIR418), and was recovered by complementation of intact VR-RNA in TS140(pSB1031) (Fig. 5B). However, at 3 h, no significant changes among the strains were found in the *hemO* mRNA amount (Figs. 5A and B). These data suggested that the *hemO* gene is positively regulated by both VirR/VirS and VR-RNA, especially at 2 h of culture (the rapidly growing phase of *C. perfringens*) and that *hemO* belongs to the VirR/VirS-VR-RNA regulon in *C. perfringens*.

In conclusion, characterization of CPE0214 (*hemO*) revealed that it seems to play a role in heme utilization in the anaerobic pathogen *C. perfringens*. Other than virulence-associated genes, the VirR/VirS-VR-RNA regulon includes a variety of genes whose functions are closely related to cell survival in the host (Ohtani *et al.*, 2009). In particular, VirR/VirS-VR-RNA-mediated control of the genes required for metabolizing extracellular sources is very important for cell survival and multiplication. Here, our data indicate that the VirR/VirS-VR-RNA system is also important for the regulation of *hemO*, which might be required for the acquisition of iron and the maintenance of an anoxic environment to allow survival in a host environment. However, we have very little knowledge about the iron metabolism in *C. perfringens*. Further detailed studies will thus be needed to elucidate the regulation of iron metabolism and its relationships with the VirR/VirS-VR-RNA system, which will be prerequisite for understanding the pathogenicity of *C. perfringens*.

## Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research in the Priority Area "Applied Genomics" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by Health and Labor Sciences Research Grants (Research on Food Safety).

## References


Aiba, H., S. Adhya, and B. de Crombrugghe. 1981. Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J. Biol. Chem.* 256, 11905-11910.

Ba-Thein, W., M. Lyristis, K. Ohtani, I.T. Nisbet, H. Hayashi, J.I. Rood, and T. Shimizu. 1996. The *virR/virS* locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. *J. Bacteriol.* 178, 2514-2520.

Bruggemann, H., R. Bauer, S. Raffestin, and G. Gottschalk. 2004. Characterization of a heme oxygenase of *Clostridium tetani* and its possible role in oxygen tolerance. *Arch. Microbiol.* 182, 259-263.

Hatheway, C.L. 1990. Toxigenic clostridia. *Clin. Microbiol. Rev.* 3, 66-98.

Kobayashi, T., T. Shimizu, and H. Hayashi. 1995. Transcriptional analysis of the β-galactosidase gene (*pbg*) in *Clostridium perfringens*. *FEMS Microbiol. Lett.* 133, 65-69.

Lyristis, M., A.E. Bryant, J. Sloan, M.M. Awad, I.T. Nisbet, D.L. Stevens, and J.I. Rood. 1994. Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. *Mol. Microbiol.* 12, 761-777.

McDonel, J.L. 1980. *Clostridium perfringens* toxins (Type A, B, C, D, E). *Pharmacol. Ther.* 10, 617-635.

Ohtani, K., H. Hirakawa, K. Tashiro, S. Yoshizawa, S. Kuhara, and T. Shimizu. 2009. Identification of a two-component VirR/VirS regulon in *Clostridium perfringens*. *Anaerobe* Article in press.

Okumura, K., K. Ohtani, H. Hayashi, and T. Shimizu. 2008. Characterization of genes regulated directly by the VirR/VirS system in *Clostridium perfringens*. *J. Bacteriol.* 190, 7719-7727.

Petit, L., M. Gibert, and M.R. Popoff. 1999. *Clostridium perfringens*: toxinotype and genotype. *Trends Microbiol.* 7, 104-110.

Riha, W.E., Jr. and M. Solberg. 1971. Chemically defined medium for the growth of *Clostridium perfringens*. *Appl. Microbiol.* 22, 738-739.

Rood, J.I. 1998. Virulence genes of *Clostridium perfringens*. *Annu. Rev. Microbiol.* 52, 333-360.

Sambrook, J., E.F. Fritch, and T. Maniatis. 1989. Molecular cloning. A laboratory manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, N.Y., USA.

Schmitt, M.P. 1997. Transcription of the *Corynebacterium diphtheriae hmuO* gene is regulated by iron and heme. *Infect. Immun.* 65, 4634-4641.

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

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. *Proc. Natl. Acad. Sci. USA* 99, 996-1001.

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

Studier, F.W. and B.A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189, 113-130.

Wilks, A. and M.P. Schmitt. 1998. Expression and characterization of a heme oxygenase (Hmu O) from *Corynebacterium diphtheriae*. Iron acquisition requires oxidative cleavage of the heme macrocycle. *J. Biol. Chem.* 273, 837-841.

Wyckoff, E.E., M. Schmitt, A. Wilks, and S.M. Payne. 2004. HutZ is required for efficient heme utilization in *Vibrio cholerae*. *J. Bacteriol.* 186, 4142-4151.

Yoshida, T. and G. Kikuchi. 1978. Purification and properties of heme oxygenase from pig spleen microsomes. *J. Biol. Chem.* 253, 4224-4229.

Zhang, X., M. Sato, M. Sasahara, C.T. Migita, and T. Yoshida. 2004. Unique features of recombinant heme oxygenase of *Drosophila melanogaster* compared with those of other heme oxygenases studied. *Eur. J. Biochem.* 271, 1713-1724.

Zhu, W., D.J. Hunt, A.R. Richardson, and I. Stojiljkovic. 2000. Use of heme compounds as iron sources by pathogenic neisseriae requires the product of the *hemO* gene. *J. Bacteriol.* 182, 439-447.

Zhu, W., A. Wilks, and I. Stojiljkovic. 2000. Degradation of heme in Gram-negative bacteria: the product of the *hemO* gene of Neisseriae is a heme oxygenase. *J. Bacteriol.* 182, 6783-6790.