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

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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

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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

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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
                                *** ** *** :::.* ::: *:**:: **:***:***** * .::
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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

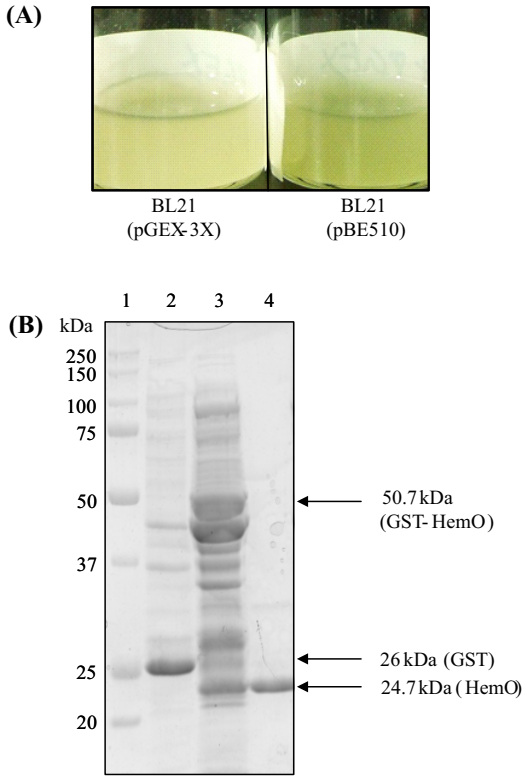

Fig. 2. Expression and purification of the CPE0214 (HemO) protein. (A) *E. coli* BL21 carrying pGEX-3X (control) and pBE510 (pGEX-3X+*hemO*) were cultured in LB with 1 mM IPTG for 8 h. Note that the medium of BL21 (pBE510) turned a pale green color. (B) Purification of HemO. Lanes: 1, protein size marker;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

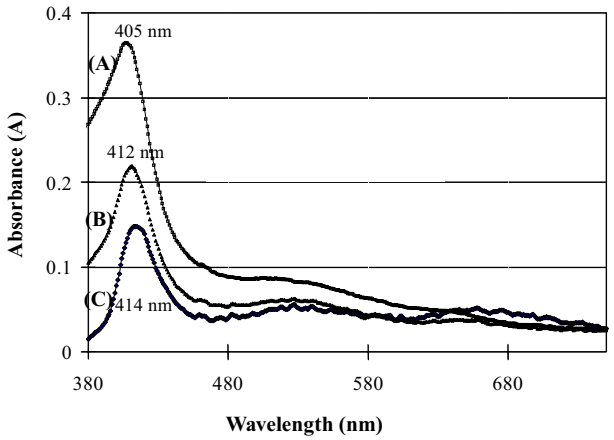

Fig. 3. Heme degradation to biliverdin catalyzed by HemO. Heme conversion was initiated by the addition of ascorbic acid (AA) to the HemO-heme complex, and spectral changes between 300 and 750 nm were recorded at 10 and 40 min. (A) HemO-heme complex (before addition of AA); (B) 10 min after AA addition; (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 *hem*O. 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 *hem*O 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

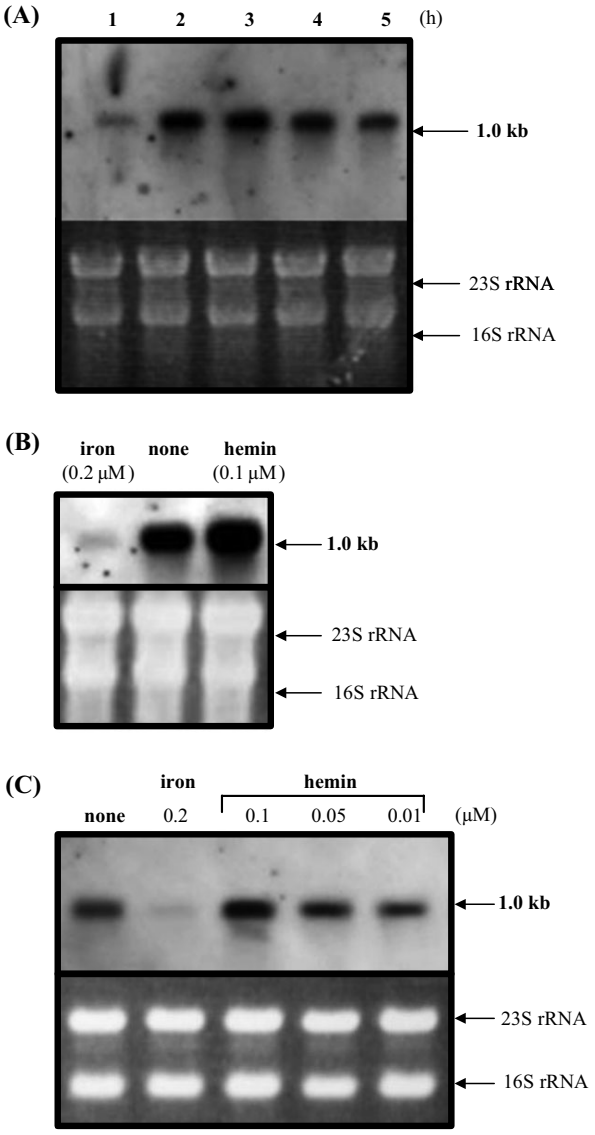

Fig. 4. Transcriptional analysis of *hemO* by Northern hybridization. (A) Growth-phase-dependent expression of the *hemO* mRNA (1 h to 5 h). The mRNA of *hemO* is indicated by an arrow along its length. (B) Transcriptional change of *hemO* in the presence of 0.2 μM iron ($FeSO_4 \cdot 7H_2O$) or 0.1 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*

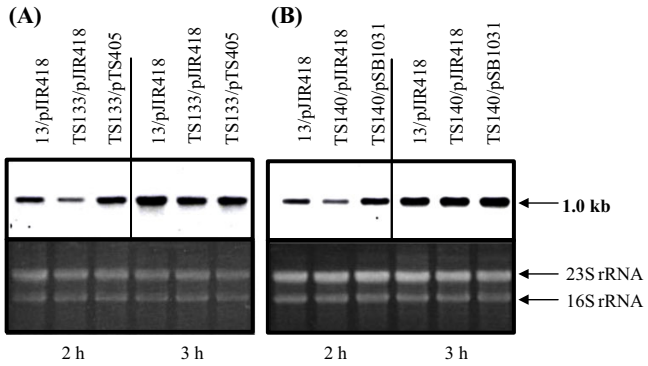

Fig. 5. Regulation of *hemO* by VirR/VirS and the VR-RNA system. Total RNA was extracted from the *C. perfringens* strains at 2 and 3 h from the start of the culture. (A) The wild-type strain 13(pJIR418), TS133(pJIR418)(*virR*$^-$), and TS133(pBT405) (*virR/virS*$^+$) were examined. (B) The wild-type 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

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