Genetic and biochemical analysis of a class C non-specific acid phosphatase (NSAP) of *Clostridium perfringens*

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Clostridium perfringens, a Gram-positive anaerobe, is a human pathogen that causes gas gangrene in muscle tissues. Its ability to grow and survive in the host is believed to be due to the production of numerous enzymes that enable the organism to obtain essential nutrients from the host. In this study, CPE0201, a putative acid phosphatase gene deduced by genome analysis, was shown to encode a non-specific acid phosphatase in *C. perfringens*. Multiple alignments of the amino acid sequence showed that CPE0201 shares two signature motifs that belong to a class C acid phosphatase family. Expression of CPE0201 was shown to be positively regulated by the global VirR/VirS-VR-RNA regulatory cascade at the transcriptional level. To determine the acid phosphatase activity of the CPE0201-encoded protein, cloning, expression, purification and several biochemical characterizations were carried out. The optimum pH for activity of the CPE0201 enzyme was 4.8, and its $V_{\max}$ and K_m were $3.08 \text{ nmol ml}^{-1} \text{ min}^{-1}$ and 2.84 mM , respectively, with *p*-nitrophenyl phosphate (PNPP) as substrate. A CPE0201 mutant did not grow in a minimal medium containing PNPP, while it showed normal growth when Na_2HPO_4 was added to the medium. The enzyme appears to be associated with the surface of the cell, where it may function to acquire inorganic phosphate from organic phosphomonoesters in acidic conditions, which could play an important role in the survival and growth of *C. perfringens* in the host tissue.

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INTRODUCTION

Clostridium perfringens is a Gram-positive anaerobic, spore-forming human pathogen that causes gas gangrene in muscle tissues. Since *C. perfringens* has been found to possess acid phosphatase activity in acidic conditions, a method to confirm the organism's presence was developed by measuring its acid phosphatase using different phosphatase reagents (Ueno *et al.*, 1970). Since then, improved methods based on acid phosphatase detection have been adapted as routine protocols to detect *C. perfringens* (Eisgruber *et al.*, 2003). Although the acid phosphatase activity has long been employed as a characteristic of *C. perfringens* that differentiates it from other clostridial species, there are still few reports about the gene(s) that contribute to the acid phosphatase profile.

Bacteria have several enzymes that can dephosphorylate organic compounds, which play various essential or accessory roles in cell physiology. Some of these enzymes can be secreted outside the cell, where they are either released in a soluble form or retained as membrane-bound proteins (Rossolini *et al.*, 1998) to hydrolyse organic

compounds into inorganic phosphate and organic byproducts that can be transported across the membrane, thus providing the cell with essential nutrients (Beacham, 1979).

Non-specific acid phosphohydrolases or phosphatases (NSAPs) are widely distributed among many Gram-positive and Gram-negative bacterial species. NSAPs are considered physiologically important because they either help the cell to utilize organic phosphoesters that cannot cross the cytoplasmic membrane (Beacham, 1979), or have functional importance in gene expression (Carmany *et al.*, 2003). NSAPs are usually grouped into three types (classes A, B and C) on the basis of amino acid sequence relatedness (Thaller *et al.*, 1994, 1995, 1997); they can dephosphorylate a broad array of structurally unrelated substrates and exhibit optimal catalytic activity at acidic to neutral pH values (Rossolini *et al.*, 1998). Class C NSAPs have recently been identified as a subgroup of secreted bacterial lipoproteins endowed with NSAP activity that contain a polypeptide component with a molecular mass of approximately 30 kDa and share conserved sequence motifs (Green *et al.*, 1991; Thaller *et al.*, 1997).

Although the whole genome sequence of *C. perfringens* has been analysed (Shimizu *et al.*, 2002a), the acid

Abbreviations: GST, glutathione S-transferase; NSAP, non-specific acid phosphatase; PNPP, *p*-nitrophenyl phosphate.

phosphatase(s) and its gene(s) in *C. perfringens* still have not been clearly revealed. However, the protein encoded by the CPE0201 gene has amino acid sequence similarities above 40% with several acid phosphatases of Gram-positive bacteria, including *Bacillus anthracis* (42.3% identity in 243 aa overlap) (Felts *et al.*, 2006). This fact prompted us to explore the possibility that CPE0201 encodes a protein with acid phosphatase activity. In this study, cloning, expression, purification, biochemical characterizations and phylogenetic analysis of CPE0201 and the protein it encodes have been employed to reveal its acid phosphatase properties.

METHODS

Strains, media and culture conditions. *C. perfringens* strains 13 (Mahony & Moore, 1976), TS133 (*virR* mutant) (Shimizu *et al.*, 1994) and TS140 (*vr* mutant) (Shimizu *et al.*, 2002b) were cultured in GAM (Gifu Anaerobic Medium; Nissui, Japan) at 37 °C under anaerobic conditions, as described previously (Shimizu *et al.*, 1994). A minimal medium that was modified from the original (Riha & Solberg, 1971) was also used for the culture of *C. perfringens* strains. *Escherichia coli* strains DH5 α and BL21 were cultured under standard conditions (Sambrook *et al.*, 1989). Plasmid pUC19 was used for general cloning in *E. coli*, and pGEX-3X was used to construct an expression vector for recombinant proteins. A GSTrap FF column (GE Science) was used to purify a glutathione S-transferase (GST) fusion protein expressed in *E. coli* BL21.

Northern hybridization. Total RNA from *C. perfringens* was extracted and Northern blotting performed as previously described (Aiba *et al.*, 1981) using an AlkPhos-direct kit and CDP-star Chemiluminescence Reagent (GE Healthcare). DNA probes were prepared by PCR from genomic DNA of *C. perfringens* strain 13 with the appropriate primer sets.

Cloning, expression and purification. To express the predicted NSAP from *C. perfringens*, the CPE0201 gene was amplified by PCR under standard cycling conditions using primers P201F (5'-ccggaatccatagaaacttcaaaaattac-3') and P201R (5'-ccgaattcttatttaattgattttatgc-3'), containing *Bam*HI and *Eco*RI restriction sites, respectively (underlined). Following digestion with *Bam*HI and *Eco*RI, the amplified DNA fragment was subcloned into pGEX-3X by using the same restriction sites. Finally, the resulting plasmid construct was used to transform competent cells of *E. coli* BL21. A single colony of *E. coli* BL21 containing the plasmid pGEX-CPE0201 was inoculated into 5 ml LB medium containing 50 μ g ampicillin ml⁻¹ and incubated overnight at 37 °C with constant aeration. A 1:100 dilution of this overnight culture was then inoculated to 200 ml fresh LB medium. The sample was cultured until the culture reached OD₆₀₀ 0.6, at which point IPTG was added to a final concentration of 1 mM. The cells were further incubated for 4 h at 25 °C after IPTG induction, then harvested by centrifugation at 5000 g for 10 min. The resulting pellet was resuspended in 10 ml PBS (pH 7.4), and centrifuged at 5000 g for 10 min. After being washed twice by PBS, the pellet was suspended in 5 ml PBS containing one tablet of Complete Mini Protease Inhibitor Cocktail (Recho). The cells were disrupted by ultrasonic treatment, and unbroken cells and pelletable debris were removed by centrifugation at 15 000 r.p.m. for 10 min. Following filtration with a 0.45 μ m filter, the resulting extract was then applied onto a GSTrap FF column. The GST-CPE0201 fusion protein was purified according to the manufacturer's instructions. The protein concentration was measured by the method of Bradford (1976), using BSA as a standard. The purified protein was checked by

12% SDS-PAGE to be of the correct molecular mass and sufficiently pure for the subsequent characterizations.

Enzyme activity assay. The acid phosphatase activity of the GST-CPE0201 product was determined colorimetrically by the release of *p*-nitrophenol from a 6 mM solution of *p*-nitrophenyl phosphate (PNPP) in 100 mM sodium acetate/acetic acid buffer, pH 4.8, containing 5 mM magnesium chloride. Normally the substrate volume was 100 μ l for a 5 μ l enzyme sample volume. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 100 μ l 1 M sodium hydroxide. Absorbance at 405 nm was measured in a spectrophotometer against a blank of reagents to which the sample was added after the sodium hydroxide. The amounts of *p*-nitrophenol released by the enzyme were determined from an appropriate calibration curve. One unit of enzyme activity was defined as the amount of enzyme able to release 1 mmol *p*-nitrophenol per min under the assay conditions.

Influences of pH and temperature. The thermal stability of the acid phosphatase activity of GST-CPE0201 was determined in a standard assay with the following modifications. Samples of purified enzyme (0.005 U of activity) in 100 μ l 100 mM sodium acetate/acetic acid buffer, pH 4.8, containing 5 mM MgCl₂, were kept at 4, 25, 37, 45, 50 or 60 °C for 30 min and returned to 4 °C for 10 min, then all the samples were assayed for enzymic activity. The pH profile of acid phosphatase of GST-CPE0201 was determined by assaying 0.005 U of enzyme at 37 °C for 30 min in sodium acetate/acetic acid buffer (pH 4.4 to 6.2) at increments of 0.2 pH unit.

Effect of inhibitors on enzyme activity. The following compounds were tested for their inhibitory effect in the standard assay at the concentrations indicated: 20 mM EDTA, 0.47 mM sodium molybdate, 0.1% SDS, 1.2 mM sodium fluoride (NaF) and 12 mM L-(+)-tartrate.

Construction of CPE0201 mutant. A 450 bp fragment was amplified from the coding region of CPE0201 by PCR using an appropriate primer set. The fragment was cloned into the *Sma*I site of plasmid pUC118 containing the *ermBP* (erythromycin-resistance) gene. The plasmid was electroporated into *C. perfringens* strain 13 as described previously (Shimizu *et al.*, 1994), and erythromycin-resistant transformants resulting from single-crossover homologous recombination were selected. The mutation of CPE0201 was confirmed by Southern hybridization using a CPE0201 gene probe.

Phylogenetic analysis. Sequence analyses of CPE0201 of *C. perfringens* were done using the BLAST algorithm on the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>). Sequence data were manipulated with different subroutines from the DNASTAR program. The multiple alignments for sequences were performed by using MEGALIGN in DNASTAR. Amino acid sequences of different acid phosphatases were aligned using MEGA4 software (Tamura *et al.*, 2007) and a phylogenetic tree constructed using the neighbour-joining method (Saitou & Nei, 1987). The 16S rDNA sequences of all bacterial species tested were aligned by the same method.

RESULTS

Comparative and phylogenetic analyses of CPE0201

BLAST searches against protein sequence databases in GenBank revealed significant levels (45–33% sequence identity) of similarity between CPE0201 and several other prokaryotic acid phosphatases. Similarities were found

with acid phosphatase from *Bacillus anthracis* (NP_846955, 40 %), *Elizabethkingia meningoseptica* (CAA73299, 41 %), *Streptococcus agalactiae* (NP_688757, 36 %), *Helicobacter pylori* (AAQ88280, 33 %), *Chlorobium phaeobacteroides* (YP_001959498, 32 %), *Porphyromonas gingivalis* (YP_001929139, 38 %) and *Anabaena variabilis* (YP_320194, 32 %). Moreover, a considerable level of similarity was also found with some eukaryotic acid phosphatases even though their functions are unknown.

Multiple alignment of the amino acid sequences among the above-mentioned bacterial phosphatases revealed that CPE0201 possesses two motifs in its amino acid sequence. One is (I/V)-(V/A/L)-D-(I/L)-D-E-T-(V/M)-L-X-(N/T)-XX-Y, near the N terminus, and the other is (I/V)-(L/M)-XX-G-D-(N/T)-L-X-D-F, near the C terminus (Fig. 1). These motifs have been described as the common signature motifs of bacterial class C NSAPs (Thaller *et al.*, 1998). Peptide sequence alignment shows that the signal peptide of CPE0201 shares significant sequence similarity with other typical bacterial lipoproteins having NSAP activity (data not shown). When we determined the acid phosphatase activities in culture supernatant and cell suspension of *C. perfringens*, the activity was only detected in the cell suspension, which implies that the enzyme is not secreted into the supernatant but is associated with the cell membrane or cell wall (data not shown).

The phylogenetic relationships between the CPE0201 protein and representative homologues in other genera of Gram-positive and Gram-negative bacteria are shown in Fig. 2(b). A phylogenetic analysis based on 16S rDNA sequences of those bacteria was also carried out (Fig. 2a). As shown in Fig. 2(a), the 24 bacteria were divided into groups by 16S rDNA sequence similarity, which showed good accordance with commonly accepted division theory; however, the groupings based on the acid phosphatase sequences were somewhat different from those based on the 16S rDNA analysis. Especially, CPE0201 seems to have a

closer relationship to some homologues in Gram-negative groups than in Gram-positive ones (Fig. 2b). This implies that CPE0201 may not be derived from an ancestral Gram-positive bacterium, but may have been acquired by horizontal gene transfer from an unknown ancestor; or the donor might be a Gram-negative organism.

Transcriptional regulation of CPE0201

In our preliminary experiment using a DNA microarray of *C. perfringens* strain 13, transcription of CPE0201 seemed to be positively regulated by the VirR/VirS system (Ba-Thein *et al.*, 1996; Shimizu *et al.*, 1994) and its secondary regulator VR-RNA encoded by *vrR* (Shimizu *et al.*, 2002b; K. Ohtani and others, unpublished results). The VirR/VirS-VR-RNA regulatory cascade plays important roles in regulating the expression of various toxin genes, which contributes to the pathogenicity of *C. perfringens*. To confirm the microarray data, we performed Northern hybridization experiments using wild-type strain 13, *virR* mutant strain TS133 and *vrR* mutant strain TS140. As shown in Fig. 3, the transcription of CPE0201 decreased in TS133 and TS140 at both 2 h and 3 h of culture (early to mid-exponential growth), while the expression of CPE0201 was restored by complementation with intact *virR/virS* and *vrR* genes in strains TS133/pTS405 and TS140/pTS1031, respectively. The higher level of expression of CPE0201 in the complemented strains than in the wild-type may be due to the overexpression of the *virR/virS* or *vrR* gene supplied by the high-copy-number plasmids. The expression pattern of CPE0201 was quite similar to that of *vrR*, encoding VR-RNA, indicating that CPE0201 is under the positive control of the VirR/VirS-VR-RNA regulatory cascade in *C. perfringens*.

Expression of CPE0201 in *E. coli*

The product of CPE0201 has 94 % identity with that of the CPF0190 protein from *C. perfringens* ATCC 13124

Fig. 1. Alignment of the amino acid sequence of CPE0201 with the amino acid sequences of various bacterial class C or class B NSAPs. Alignment was maximized by introducing gaps, which are indicated by hyphens. The numbers indicate positions from the N terminus. A majority consensus sequence (amino acid residues present in at least four of the proteins) is shown in the upper line. The regions corresponding to the fingerprint patterns for class C acid phosphatases are boxed.

Fig. 2. Phylogenetic relationship based on (a) 16S rDNA sequence and (b) the amino acid sequences of acid phosphatases from various bacteria.

(GenBank accession no. CP000246) and shows 93 % identity with the product of CPR0188 from *C. perfringens* SM101 (GenBank accession no. CP000312). The CPE0201

gene encodes a 287 aa protein whose molecular mass is 32.5 kDa, which corresponds well with the general molecular mass (30 kDa) of class C NSAPs (Rossolini *et al.*, 1998). To express the protein from CPE0201, we inserted the whole protein-coding region of CPE0201 downstream from the GST tag sequence in plasmid pGEX-3X. A heavily Coomassie-stained band of approximately 56 kDa from IPTG-induced cells was found by SDS-PAGE. Purification of the recombinant GST-CPE0201 fusion protein was performed by using a GSTrap affinity column, and the purified product was confirmed by 12 % SDS-PAGE (Fig. 4). When we checked the acid phosphatase activity of the GST-CPE0201 fusion protein and the CPE0201 protein with the GST tag removed, there was no obvious difference between the two (data not shown); therefore, the GST-CPE0201 fusion protein was used for all the subsequent experiments.

Fig. 3. Northern analysis of the expression of CPE0201. Total RNA was prepared from 2 h and 3 h cultures of *C. perfringens*, and subjected to Northern hybridization. Ten micrograms of total RNA was loaded in each lane (loading was confirmed in ethidium-bromide-stained gel). Gene probes used for hybridization are indicated on the left. Representative data from triplicate experiments that showed similar results are presented.

Acid phosphatase activity of CPE0201 product

The K_m and V_{max} of the GST-CPE0201 enzyme for PNPP were determined in 100 μ l assay mixture containing 0.005 U of enzyme by measuring the release of *p*-nitrophenol for 30 min at 12 different substrate concentrations (0.1–10 mM). The rate of substrate hydrolysis was found to follow Michaelis–Menten kinetics at low substrate concentrations (0.1–3 mM). The values obtained for V_{max} and K_m from a Lineweaver–Burk double reciprocal plot were 3.08 nmol ml⁻¹ min⁻¹ and 2.84 mM, respectively.

Fig. 4. Expression of GST-CPE0201 fusion protein in *E. coli*. The SDS-PAGE gel was stained with Coomassie blue. Lanes: 1, *E. coli* lysate prior to IPTG induction; 2, *E. coli* lysate after IPTG induction; 3, purified GST-CPE0201 fraction; lane 4, protein molecular mass standards.

Limited thermal stability of CPE0201 product

To check the thermal stability of the NSAP activity of GST-CPE0201 fusion protein, a sample containing 0.005 U of GST-CPE0201 enzymic activity was heated at different temperatures for 30 min and then its NSAP activity was measured. This activity was optimum at 40 °C but dropped rapidly by 78 % at 50 °C and by 84 % at 55 °C (data not shown). These data indicate that the NSAP activity of CPE0201 shows limited thermal stability and that the optimum temperature differs from those reported for several other class C NSAPs (Thaller *et al.*, 1998).

Effect of pH on NSAP activity of CPE0201 product

The NSAP activity of CPE0201 had an optimum pH of 4.8 in the standard assay (Fig. 5). It retained only 27 % of the optimal activity at pH 4.4, although this rapidly increased to 60 % at pH 4.6. It retained 94 % of optimal activity at pH 5.0, but the activity dramatically dropped to 67 % at pH 5.2. These data clearly demonstrated the acid phosphatase nature of CPE0201 protein. Unexpectedly, there was an obvious increase in the activity at pH 5.6; it retained 84 % of optimal activity, followed by a rapid decrease at pH > 5.8.

Inhibitor assays

Quantitative *in vitro* NSAP activity assays (Fig. 6) indicated that 5 mM MgCl₂ or 2 % Triton X-100 could enhance the enzyme activity, while the enzyme did not appear to be inhibited by 20 mM EDTA, 0.47 mM sodium molybdate, 12 mM L-(+)-tartrate or 1 mM sodium azide. However, 1.2 mM NaF or 0.1 % SDS significantly inhibited the enzyme activity.

Fig. 5. pH optimum assay of CPE0201 acid phosphatase activity. The pH profile of acid phosphatase of GST-CPE0201 was determined by assaying 0.005 U of enzyme at 37 °C for 30 min in sodium acetate/acetic acid buffer at various pH values. Means $\pm$ SD ($n=3$) are plotted.

Analysis of CPE0201 mutant

We constructed a CPE0201 mutant (RWM0201) from *C. perfringens* strain 13 by homologous recombination using the internal DNA fragment of CPE0201. Cell suspensions of RWM0201 did not show any acid phosphatase activity (data not shown). To investigate the importance of CPE0201 acid phosphatase in the physiology of *C. perfringens*, we examined the growth of RWM0201 in the presence of PNPP or inorganic phosphate in a minimal

Fig. 6. Effects of various inhibitors on CPE0201 acid phosphatase activity. Activity was determined in the standard assay at 37 °C, with 0.005 U of enzyme, 6 mM PNPP and the corresponding inhibitor; values (means $\pm$ SD, $n=3$) are shown relative to a control with no inhibitor.

medium lacking other sources of phosphate (Fig. 7). In the minimal medium containing 1 mM Na_2HPO_4 , both strain 13 (wild-type) and RWM0201 grew similarly, while RWM0201 could not grow in the medium containing PNPP only. These data indicate that the CPE0201 enzyme is important in degrading organic phosphomonoesters into inorganic phosphate in environments where no inorganic phosphate is available and that CPE0201 is the only gene encoding acid phosphatase in *C. perfringens*.

Distribution of CPE0201 among *C. perfringens* strains

To investigate the importance of CPE0201 in *C. perfringens*, we checked the existence of the gene in other strains of *C. perfringens* stocked in our laboratory (four type A strains, two strains each from type B, type C, type D and type E). A PCR-based analysis using the CPE0201 primer set detected identical DNA fragments in all 12 strains (data not shown). These data indicate that CPE0201 is highly conserved among various *C. perfringens* strains, which may imply the importance of acid phosphatase in the physiology of *C. perfringens*.

DISCUSSION

Numerous extracellular toxins and enzymes of *C. perfringens* are believed to be involved in the ability to cause gas gangrene (clostridial myonecrosis) in humans, including alpha-, theta- and kappa-toxins (Rood, 1998). The role of the acid phosphatase encoded by CPE0201 in the physiology of the organism is also of great interest to us because, in present study, the transcription of CPE0201 was shown to be regulated by the VirR/VirS-VR-RNA cascade, which also involves the genes for alpha-, kappa- and theta-toxins (Ba-Thein *et al.*, 1996). The genetic mechanism of the global regulation by VR-RNA is still unclear, since the

genes regulated by VR-RNA share no common DNA motifs in their promoter or leader regions. Similarly, there was no apparent DNA sequence in the promoter region of CPE0201 that was similar to those of other VR-RNA-regulated genes.

Classification of bacterial phosphatases was initially based on the biochemical and biophysical properties of the enzymes, such as pH optimum (acid, neutral or alkaline), substrate profile (non-specific vs specific for certain substrates) and molecular size (high vs low molecular mass) (Rossolini *et al.*, 1998). As numerous nucleotide sequence data became available, phosphatases were grouped into different molecular families according to similarity at the level of the primary structure. In this study, multiple alignments of the deduced amino acid sequence of the CPE0201 protein against nonredundant protein databases in GenBank showed that CPE0201 shares two conserved signature motifs belonging to an acid phosphatase family. It shows the greatest homology to several acid phosphatases of class C bacterial NSAPs. Furthermore, we have shown here that the CPE0201 protein has typical NSAP biochemical properties. To our knowledge, CPE0201 is the first clostridial acid phosphatase to be identified and characterized at a biochemical level. Based on phylogenetic and structural considerations, we propose that the CPE0201 should be defined as a member of the class C NSAPs, a broad group of phosphatases consisting of secreted proteins that function in an acidic-to-neutral pH range and that use a wide variety of organic phosphoesters as substrates (Rossolini *et al.*, 1998). It is possible that in the normal habitat of *C. perfringens*, the CPE0201 acid phosphatase may play a physiological role as a scavenger in rendering organic phosphomonoesters available for transport into the cell; otherwise, they cannot pass through the cytoplasmic membrane (Dissing & Uerkvitz, 2006).

Fig. 7. Growth curves of the wild-type strain (◆, solid lines) and the CPE0201 mutant RWM0201 (▲, dashed lines) of *C. perfringens* at 37 °C, in modified minimal medium with 1 mM Na_2HPO_4 (a) or 0.1 mM PNPP (b).

Based on a functional search against all ORFs in the whole genome of *C. perfringens* strain 13, CPE0201 is believed to represent the only gene to encode an NSAP. Generally, acid phosphatases of Gram-positive bacteria are bound to the cell surface (Okabayashi *et al.*, 1974) or cytoplasmic membrane (Poirier & Holt, 1983). CPE0201 appears to be the only surface-associated acid phosphatase of *C. perfringens*. However, the precise location of CPE0201 at a subcellular level should be further investigated. Whether or not the enzyme is produced in the human host suffering from *C. perfringens* infection is presently unknown, and therefore its exact pathological importance to humans should be examined.

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