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

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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 (Carmy 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
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 (*vrB* mutant) (Shimizu et al., 2002b) were cultured in GAM (Gifu Anaerobic Medium; Nissui, Japan) at 37°C anaerobic conditions, as described previously (Shimizu et al., 1994). Strains of *E. coli* with appropriate restriction sites. Finally, the resulting plasmid construct was purified according to the manufacturer's instructions.

Cloning, expression, purification, and biochemical characterization. The protein was purified according to the manufacturer's instructions. The purified protein was checked by 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.

**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, *vrr* 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 *vrr*/*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 CEP0201 in *E. coli***

The product of CEP0201 has 94% identity with that of the CPF0190 protein from *C. perfringens* ATCC 13124.
(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.

**Acid phosphatase activity of CPE0201 product**

The $K_m$ and $V_{max}$ of the GST-CPE0201 enzyme for PNPP were determined in 100 µl assay mixture containing 0.005 U of enzyme by measuring the release of p-nitrophenol for 30 min at 12 different substrate concentrations (0.1–30 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.

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

**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.
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.

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...
medium lacking other sources of phosphate (Fig. 7). In the minimal medium containing 1 mM Na₂HPO₄, 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 Na₂HPO₄ (a) or 0.1 mM PNPP (b).](image-url)
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 (Oyabayashi 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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