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

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

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

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 phylogenic 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 (virA 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 used for the culture of *C. perfringens* strains. *Escherichia coli* strains DH5α 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’-cgcgtccattacaagctttaaatac-3’) and P201R (5’-cgcgtccattacaagctttaaatac-3’), containing BamHI and EcoRI restriction sites, respectively (underlined). Following digestion with BamHI and EcoRI, 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 μg 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 0.1 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 15000 r.p.m. for 10 min. Following filtration with a 0.45 μm 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/acetate buffer, pH 4.8, containing 5 mM magnesium chloride. Normally the substrate volume was 100 μl for a 5 μl enzyme sample volume. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 100 μl 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 μl 100 mM sodium acetate/acetate 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/acetate 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 i-(- +) ittrate.

**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 Smal 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 MEGAS4 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 (CAAA73299, 41 %), Streplococcus 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 CEP0201 in E. coli

The product of CEP0201 has 94 % identity with that of the CPF0190 protein from C. perfringens ATCC 13124
The 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 (0.1–10 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.
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.
medium lacking other sources of phosphate (Fig. 7). In the minimal medium containing 1 mM Na$_2$HPO$_4$, 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 phosphoesters 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$_2$HPO$_4$ (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 (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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