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Expression, purification and crystallization of an atypical class C acid phosphatase from *Mycoplasma bovis*

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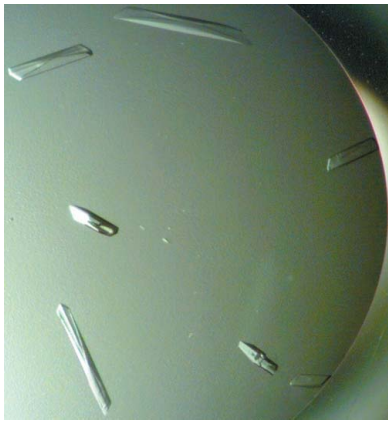
Class C acid phosphatases (CCAPs) are 25–30 kDa bacterial surface proteins that are thought to function as broad-specificity 5′,3′-nucleotidases. Analysis of the newly published complete genome sequence of *Mycoplasma bovis* PG45 revealed a putative CCAP with a molecular weight of 49.9 kDa. The expression, purification and crystallization of this new family member are described here. Standard purification procedures involving immobilized metal-ion affinity chromatography and ion-exchange chromatography yielded highly pure and crystallizable protein. Crystals were grown in sitting drops at room temperature in the presence of PEG 3350 and HEPES buffer pH 7.5 and diffracted to 2.3 Å resolution. Analysis of diffraction data suggested a primitive monoclinic space group, with unit-cell parameters a = 78, b = 101, c = 180 Å, β = 92°. The asymmetric unit is predicted to contain six molecules, which are likely to be arranged as three dimers.

1. Introduction

Phosphatases are ubiquitous enzymes that catalyze the transfer of the phosphoryl group of a phosphomonoester to water (Vincent *et al.*, 1992; Vincent & Crowder, 1995). They play important roles in many critical biological processes, including the regulation of complex signal transduction pathways, nucleotide metabolism, bone metabolism, antinociception, NAD^+ utilization and the generation, acquisition and mobilization of inorganic phosphate.

Acid phosphatases, as the name implies, are optimally active at acidic pH. Multiple classes of acid phosphatases have been identified, including histidine acid phosphatases (Rigden, 2008), purple acid phosphatases (Olczak *et al.*, 2003), alkaline phosphatase-like acid phosphatases (Felts, Reilly & Tanner, 2006) and bacterial nonspecific acid phosphatases (Rossolini *et al.*, 1998; Thaller *et al.*, 1998). The latter category includes class C acid phosphatases (CCAPs), the subject of this report.

First recognized as a group of related enzymes by Thaller *et al.* (1998), CCAPs are membrane-anchored proteins that are distinguished by several conserved properties. At the primary-structure level, CCAPs have a bipartite signature motif of (I/V)-(V/A/L)-**D**-(I/L)-**D**-E-T-(V/M)-L-*X*-(N/T)-*X*-*X*-Y near the N-terminus and (I/V)-(L/M)-*X*-*X*-G-**D**-(N/T)-L-*X*-**D**-F near the C-terminus. The four essential Asp residues embedded in the motif (bold) identify CCAPs as DDDD-superfamily phosphohydrolases. Other shared attributes include a polypeptide size of 25–30 kDa, a dimer oligomeric state in solution and the requirement of a metal cation for catalytic activity. Several class C enzymes have been characterized, including those from *Haemophilus influenzae* (Reilly *et al.*, 1999; Reilly & Smith, 1999), *Bacillus anthracis* (Felts, Reilly, Calcutt *et al.*, 2006), *Streptococcus equisimilis* (Malke, 1998), *Staphylococcus aureus* (du Plessis *et al.*, 2002), *Helicobacter pylori* (Reilly & Calcutt, 2004), *Elizabethkingia meningoseptica* (Passariello *et al.*, 2003) and *Clostridium perfringens* (Reilly *et al.*, 2009; Wang *et al.*, 2010)



Crystal structures have been reported for the CCAPs from *Haemophilus influenzae* (also known as P4; Felts *et al.*, 2007; Singh *et al.*, 2010) and *Pasteurella multocida* (Singh *et al.*, 2011). The structures showed that CCAPs belong to the haloacid dehalogenase structural superfamily, revealed the roles of the four conserved Asp residues and active-site Mg^{2+} ion and provided an understanding of substrate recognition.

Here, we report the crystallization of the CCAP from *Mycoplasma bovis*. This new family member is of interest because of its long polypeptide-chain length, which is almost twice that of other CCAPs.

2. Methods and results

2.1. Identification of the MbCCAP gene and subcloning

A query of the complete genome sequence of *M. bovis* PG45 (Wise *et al.*, 2011) with CCAP genes identified an ORF that is predicted to encode a CCAP (MbCCAP; NCBI RefSeq No. YP_004056356.1). The ORF encodes 463 residues. Multiple sequence alignments calculated using *ClustalW*2 show that MbCCAP has 18–25% pairwise amino-acid sequence identity to known CCAPs (Fig. 1). Furthermore, the alignments indicate the presence of the bipartite sequence common to CCAPs (green bars in Fig. 1). The MbCCAP polypeptide is considerably longer than those of other CCAPs (Fig. 1). The extra residues appear as six insertions scattered throughout the polypeptide chain.

CCAPs are secreted and are typically anchored to the outermost membrane by a noncleaved signal sequence or an N-terminal lipidated Cys residue; therefore, the MbCCAP sequence was analyzed for signal peptides. Analysis with the *SignalP* 3.0 (Bendtsen *et al.*, 2004), *PSORT* 6.4 (Nakai & Kanehisa, 1991), *PrediSi* (Hiller *et al.*, 2004) and *Signal-BLAST* (Frank & Sippl, 2008) servers suggests that the encoded protein contains a signal peptide with a cleavage site between Thr26 and Cys27 (black triangle in Fig. 1). The signal peptide has Lys/Arg residues at positions −19, −22 and −23 relative to Cys27, followed by a hydrophobic region, and ends in the noncanonical lipoprotein box IAAT-C. These results suggest that the protein is expressed as a precursor polypeptide that is exported from the cytoplasm and cleaved to yield a 437-residue 49.9 kDa mature enzyme. Furthermore, it is predicted that Cys27 is lipidated. It is

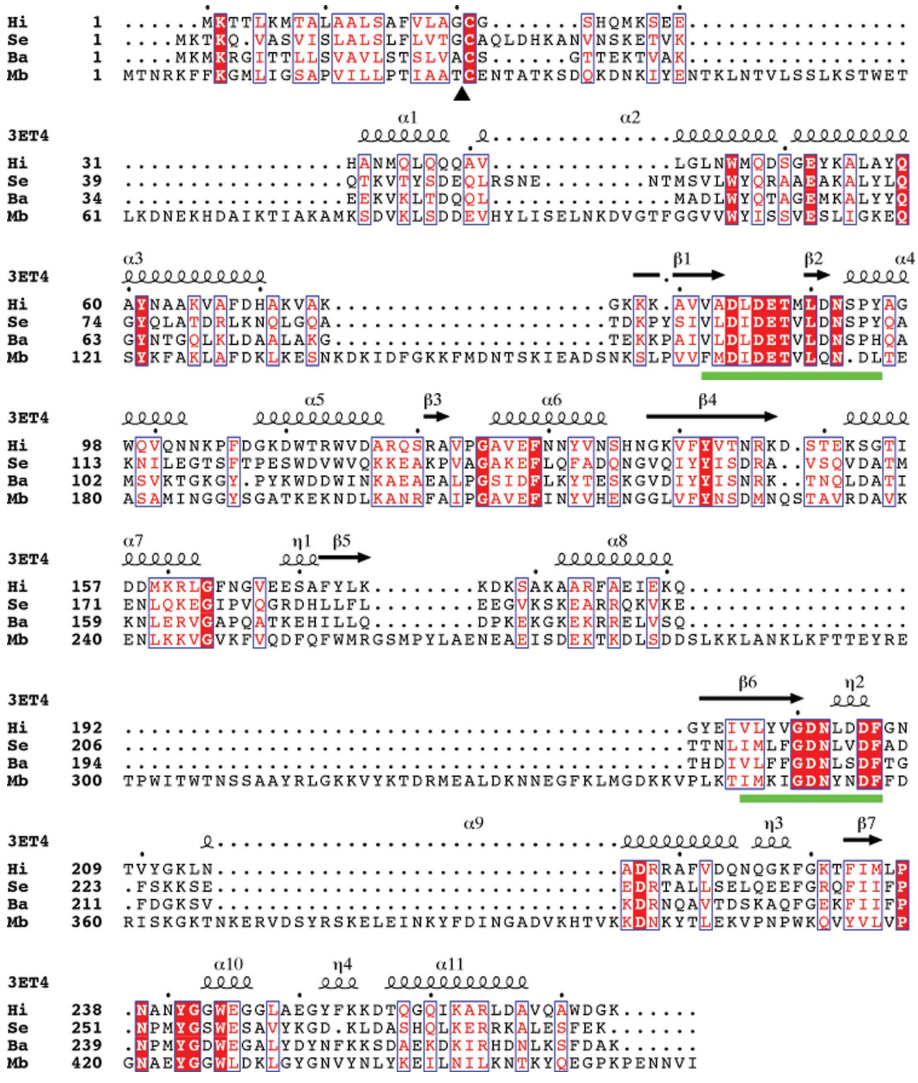


Figure 1
Sequence alignment of the CCAPs from *Haemophilus influenzae* (Hi), *Streptococcus equisimilis* (Se), *Bacillus anthracis* (Ba) and *Mycoplasma bovis* (Mb). The black triangle denotes the predicted cleavage site. The green bars denote the CCAP signature-sequence motif. The secondary-structure elements were obtained from the *H. influenzae* structure (PDB entry 3et4). This figure was prepared with *ClustalW*2 and *ESPript* (Gouet *et al.*, 1999).

noted that Cys27 aligns with cysteine residues of other CCAPs, including the Cys of P4 which is known to be lipidated (Reilly & Smith, 1999; Fig. 1). The sequence similarity to other CCAPs, bipartite DDDD motif, predicted subcellular localization and possible lipidation are consistent with classification of the enzyme into the CCAP family.

A synthetic gene encoding MbCCAP was obtained from Bio Basics Inc. (Markham, Ontario, Canada). The gene was subcloned as an *Nco*I–*Xho*I fragment into pET20b such that the predicted signal peptide is replaced by the *pelB* leader sequence and Cys27 is replaced by Met. The mature protein is exported to the *Escherichia coli* periplasm and has a C-terminal His tag consisting of Leu-Glu-His_6. The sequence of the synthetic gene has been deposited in GenBank under accession No. JN248385.

2.2. Expression and purification

The aforementioned plasmid was transformed into *E. coli* BL21 (AI). The resulting transformants were plated on MDG_{amp} plates and incubated overnight at 310 K. A single colony was picked and used to inoculate 1 l culture. The protein was expressed *via* autoinduction (Studier, 2005) at 310 K with constant shaking at 300 rev min^{-1}. The cells were harvested by centrifugation at 2681*g* for 30 min at 277 K and resuspended in 20 m*M* phosphate, 20 m*M* imidazole, 500 m*M* NaCl pH 7.0. The cell pellet was flash-cooled in liquid nitrogen and stored at 193 K.

Frozen cells were thawed at 277 K and ruptured using a French press at 6.9 MPa. Cell debris and unbroken cells were removed by centrifugation at 26 891*g* for 30 min followed by ultracentrifugation at 183 960*g* for 1 h. The resulting supernatant was loaded onto an immobilized metal-ion affinity chromatography column (Ni^{2+}-charged HiTrap, GE Healthcare) that had been equilibrated in 20 m*M* phosphate, 20 m*M* imidazole, 500 m*M* NaCl pH 7.0. The column was eluted with the equilibration buffer supplemented with 300 m*M* imidazole. Fractions that exhibited acid phosphatase activity with *p*-nitrophenylphosphate as the substrate were pooled and dialyzed overnight at 277 K into 50 m*M* sodium acetate, 2.5 m*M* $MgCl_2$, 5%(*v*/*v*) glycerol pH 5.7 (buffer *A*). The resulting sample was loaded onto a cation-exchange column (HiTrap SP, GE Healthcare) that had been equilibrated in buffer *A*. MbCCAP was eluted with a linear gradient of 0–1 *M* NaCl over 50 column volumes. The fractions were analyzed using SDS–PAGE and the purest fractions were pooled and dialyzed into 50 m*M* sodium acetate, 50 m*M* NaCl, 2.5 m*M* $MgCl_2$, 5%(*v*/*v*) glycerol pH 5.7. The sample was concentrated to 5–6 mg ml^{-1} using a centrifugal concentrating device with a 30 kDa cutoff membrane. The protein concentration was estimated using the BCA method (Pierce kit).

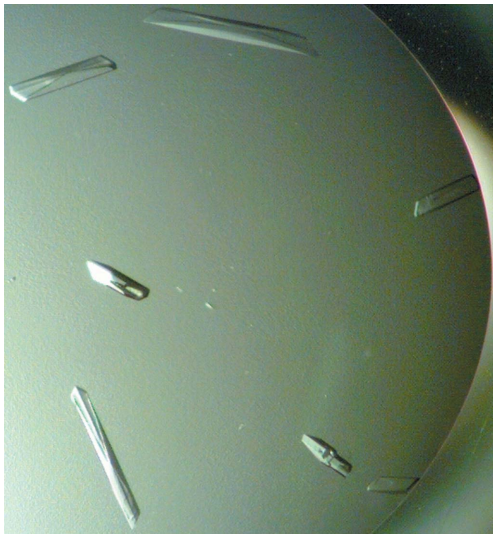


Figure 2
Crystals of MbCCAP grown in a sitting drop.

Table 1
Data-processing statistics.

Values in parentheses are for the outer resolution shell.

Space group	*P*2 or $P2_1$
Wavelength (Å)	1.0000
Unit-cell parameters (Å, °)	*a* = 78.0, *b* = 101.1, *c* = 180.5, β = 91.9
Protein molecules in asymmetric unit	6
V_M ($Å^3$ Da^{-1})	2.37
Solvent content (%)	48
Resolution (Å)	45.10–2.30 (2.38–2.30)
Total observations	442544
Unique reflections	124090
Multiplicity	3.57 (3.39)
Completeness (%)	99.6 (99.0)
Mean $I/\sigma(I)$	8.1 (2.1)
R_{merge}†	0.073 (0.416)
R_{merge} in low-resolution bin	0.033

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations of reflection *hkl*.

2.3. Crystallization and preliminary analysis of X-ray diffraction data

Crystallization trials were performed at 293 K using the sitting-drop vapor-diffusion method with Cryschem plates and reservoir volumes of 1 ml. Drops were formed by mixing 2 µl protein stock solution and 2 µl reservoir solution. Crystal screening trials using commercially available reagent kits were used to identify promising crystallization conditions. Optimization of the leads resulted in the growth of diffraction-quality crystals using reservoir solutions consisting of 0.1–0.2 *M* HEPES pH 7.5 and 22–27%(*w*/*v*) PEG 3350. The optimized crystals appeared as rods with maximum dimensions of approximately 0.6 mm in length and 0.08 mm thickness (Fig. 2). In preparation for low-temperature data collection, the crystals were soaked in 28–30% PEG 3350, 25% PEG 200, 0.1 *M* HEPES pH 7.5. The cryoprotected crystals were picked up with Hampton loops and plunged into liquid nitrogen.

The crystals were analyzed on Advanced Light Source beamline 4.2.2 using a NOIR-1 CCD detector. Autoindexing calculations with *d*TREK* (Pflugrath, 1999) suggested a primitive monoclinic lattice, with unit-cell parameters *a* = 78, *b* = 101, *c* = 180 Å, β = 92°. Using the method of Matthews (1968), the asymmetric unit was predicted to contain six MbCCAP molecules and 48% solvent (V_M = 2.37 $Å^3$ Da^{-1}). Since CCAPs are known to form dimers in solution, the six molecules in the asymmetric unit are likely to be arranged as three dimers.

A data set consisting of 719 frames was collected with a crystal-to-detector distance of 160 mm, an oscillation width of 0.25° per frame and an exposure time of 5 s per frame. The data set was integrated and scaled to 2.3 Å resolution with *d*TREK*. Data-processing statistics are listed in Table 1. Scaling of the data confirmed 2/*m* as the likely Laue class, as indicated by acceptable R_{merge} values of 0.033 in the lowest resolution bin, 0.416 in the highest resolution bin and 0.073 overall. Molecular-replacement calculations using search models derived from the coordinates of P4 (PDB entry 3et4; Felts *et al.*, 2007) and *B. anthracis* CCAP (PDB entry 2i33; R. L. Felts & J. J. Tanner,

unpublished work) did not yield a satisfactory solution; therefore, the structure of MbCCAP will be solved by experimental phasing.

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