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Purification and crystallization of a putative transcriptional regulator of the benzoate oxidation pathway in *Burkholderia xenovorans* LB400

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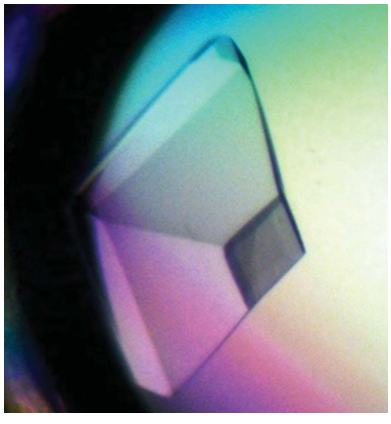

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Burkholderia xenovorans LB400 harbours two paralogous copies of the recently discovered benzoate oxidation (*box*) pathway. While both copies are functional, the paralogues are differentially regulated and flanked by putative transcriptional regulators from distinct families. The putative LysR-type transcriptional regulator (LTTR) adjacent to the megaplasmid-encoded *box* enzymes, Bxe_C0898, has been produced recombinantly in *Escherichia coli* and purified to homogeneity. Gel-filtration studies show that Bxe_C0898 is a tetramer in solution, consistent with previously characterized LTTRs. Bxe_C0898 crystallized with four molecules in the asymmetric unit of the $P4_32_12/P4_12_12$ unit cell with a solvent content of 61.19%, as indicated by processing of the X-ray diffraction data. DNA-protection assays are currently under way in order to identify potential operator regions for this LTTR and to define its role in regulation of the *box* pathway.

1. Introduction

Aromatic compounds comprise a diverse ubiquitous group of organic compounds that range from naturally occurring products such as lignin to a vast array of synthetic compounds used in agricultural, pharmaceutical and consumer products. The inherent thermodynamic stability of the aromatic ring restricts nonbiological turnover and consequently the mineralization of these compounds relies on microbial metabolic pathways. Exploiting such pathways for bioremediation purposes often entails a detailed understanding of bacterial physiology and metabolic regulation for optimal orchestration of gene expression. The potent PCB-degrading organism *Burkholderia xenovorans* LB400 (referred to hereafter as LB400) hosts an exceptionally rich metabolic inventory encoded by its 9.73 Mbp genome and presents a unique opportunity for understanding the 'pathway redundancies' observed in large-genome bacteria (Chain *et al.*, 2006). A particularly interesting feature of LB400 is that it harbours two paralogous copies of the recently discovered benzoate oxidation (*box*) pathway (box_C, chromosome 1; box_M, megaplasmid) in addition to the well characterized *ben-cat* pathway (Denef *et al.*, 2004). Both the *ben-cat* and *box* pathways are involved in the aerobic degradation of benzoate; however, the two pathways use fundamentally different mechanisms. Notably, both benzoate and benzoyl-coenzyme A (the first intermediate of the *box* pathway) are key central metabolites, forming a common conduit for various peripheral pathways including those involved in the catabolism of xenobiotic aromatic compounds. Gene-deletion experiments have confirmed that both *box* paralogues are functional (Denef *et al.*, 2006); however, characterization of the benzoate-coenzyme A ligases (the first committed step of the *box* pathway) suggests that box_M is catalytically more efficient (Bains & Boulanger, 2007). Furthermore, the two paralogues are flanked by putative regulators from two separate families and appear to be differentially regulated. Transcriptomic and proteomic studies reveal that box_C is preferentially expressed during growth on biphenyl, while box_M is expressed during the transition to stationary phase during growth on benzoate (Denef *et al.*, 2004, 2005). The precise mechanisms of regulation remain unknown: no effector or inducer molecules for either of the regulators have been proposed, nor have the respective DNA-binding sequences been defined.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P4_32_12/P4_12_12$
Unit-cell parameters (Å)	a = 112.64, c = 287.61
Wavelength (Å)	1.215
Resolution (Å)	50.38–2.50 (2.64–2.50)
Measured reflections	1443597
Unique reflections	63931
Redundancy	22.6 (22.0)
Completeness	98.4 (95.7)
$\langle I/\sigma(I)\rangle$	22.4 (8.2)
$R_{\rm merge}$† (%)	0.051 (0.494)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I_{hkl}\rangle$ is the average of symmetry-related observations of a unique reflection.

In this study, we report the production, purification and preliminary crystallization of the LysR-type transcriptional regulator (LTTR) encoded adjacent to the aldehyde dehydrogenase, benzoate-coenzyme A ligase and putative lactonase of the box_M operon of LB400 in an attempt to define a potential regulatory role for the box_M operon. A key approach in our study is to determine the high-resolution crystal structure in order to define the DNA-binding and inducer-binding regions. Although LTTRs constitute one of the largest prokaryotic regulator families (Tropel & van der Meer, 2004), relatively few structures of the full-length protein exist (Muraoka *et al.*, 2003; Lonneborg *et al.*, 2007), with the majority including only the inducer-binding domain (IBD). Ultimately, interpretation of the full-length Bxe_C0898 structure will provide a better understanding of transcriptional regulation of the *box* pathway and of transcriptional regulation in general.

2. Materials and methods

2.1. Cloning, expression and purification

The *bxe_C0898* gene was amplified from *B. xenovorans* LB400 genomic DNA and cloned into pET-28a (Novagen) in frame with an N-terminal hexahistidine tag. Sequence analysis confirmed that no mutations were introduced during amplification. Expression of recombinant *bxe_C0898* was performed in *Escherichia coli* BL21 Star (DE3) cells (Invitrogen) grown in Overnight Express Instant TB media (EMD Chemicals) supplemented with 5 µg ml^{-1} kanamycin (Sigma). After 22 h, the cells were harvested by centrifugation at 8000 rev min^{-1} for 15 min and the pellet was resuspended in buffer *A* (20 m*M* HEPES pH 8.3, 15 m*M* imidazole, 3 m*M* β-mercaptoethanol, 1 *M* NaCl, 1% glycerol). The cells were lysed using a French press (SLM Instruments) and the insoluble fraction was removed by centrifugation at 16 000 rev min^{-1} for 45 min. The supernatant containing the soluble protein was loaded onto an FPLC HisTrap FF Ni-affinity column (GE Healthcare), eluted with a concentration gradient to buffer *B* (20 m*M* HEPES pH 8.3, 1 *M* NaCl, 500 m*M* imidazole, 3 m*M* β-mercaptoethanol, 1% glycerol) and analyzed by SDS–PAGE. Fractions containing Bxe_C0898 were pooled, concentrated using Amicon Ultra (Millipore) centrifugal filter devices and thrombin-digested to remove the hexahistidine tag, resulting in a Gly-Ser-His N-terminal extension. Gel filtration was used as the final purification step using an FPLC Superdex 200 Hi-Load 16/60 size-exclusion column (GE Healthcare) in buffer *C* (20 m*M* HEPES pH 8, 150 m*M* NaCl, 3 m*M* β-mercaptoethanol, 1% glycerol). The elution volume of Bxe_C0898 was then compared with the elution profile of protein standards in order to investigate the possibility of multimerization. The fractions obtained following gel filtration were pooled based on purity and concentrated to 12 mg ml^{-1} as determined by the A_{280}.

2.2. Crystallization, data collection, processing and structure solution

Initial crystallization trials were set up with commercial screens (Wizard I, II and III from Emerald BioSystems, Index and PEG/Ion Screens from Hampton Research and Proplex from Molecular Dimensions) in 96-well plates (EMD Biosciences). The final sitting drops consisted of 1.5 µl protein solution (12 mg ml^{-1} in 20 m*M* HEPES pH 8, 150 m*M* NaCl, 3 m*M* β-mercaptoethanol, 1% glycerol) and 1.5 µl reservoir solution and were equilibrated against 100 µl reservoir solution. Small crystals of native Bxe_C0898 were observed in drops with reservoir solution consisting of 11% PEG 6000 with 100 m*M* Tris pH 8.5 after 3 d at 293 K. The use of the Hampton Research Additive Screen with this condition yielded crystals with 100 m*M* CsCl in the reservoir solution. Further refinements yielded crystals in sitting drops with 10% PEG 6000, 90 m*M* Tris pH 8.5, 250 m*M* CsCl and 10% glycerol to enable cryopreservation in the 100 µl reservoir solution. Streak-seeding was required to sufficiently improve the crystal size and quality for diffraction experiments.

A single native Bxe_C0898 crystal was looped, stepped into cryoprotectant consisting of reservoir solution supplemented with 5, 15 and 25% glycerol for 30 s and flash-cooled directly in the cryostream (100 K). Diffraction data for Bxe_C0898 were collected on beamline 9-2 at the Stanford Synchrotron Radiation Laboratory (SSRL) at a wavelength of 1.215 Å using a MAR CCD detector and 1° oscillations collected over 360°. Data processing was carried out using *CrystalClear*/*d*TREK* (Pflugrath, 1999). Data-collection statistics are presented in Table 1.

3. Results and discussion

The LTTR of the box_M pathway from LB400 was successfully cloned, produced in *E. coli*, purified and crystallized. The elution profile of Bxe_C0898 from a Superdex 200 size-exclusion column showed the regulator to be a tetramer in solution with a molecular weight of

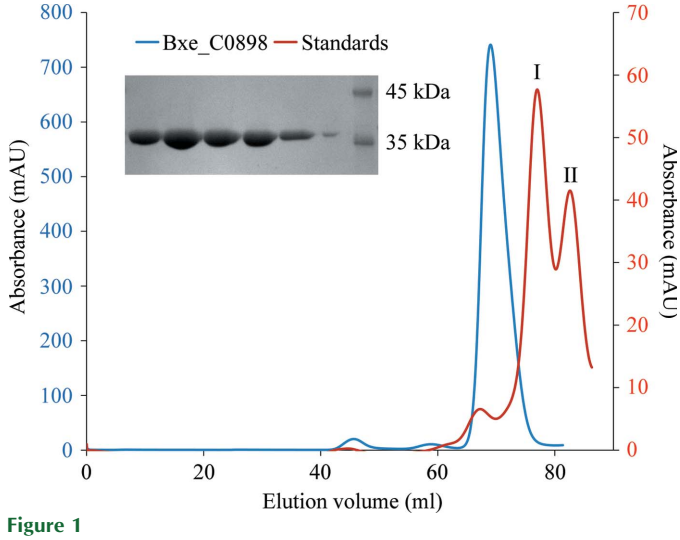
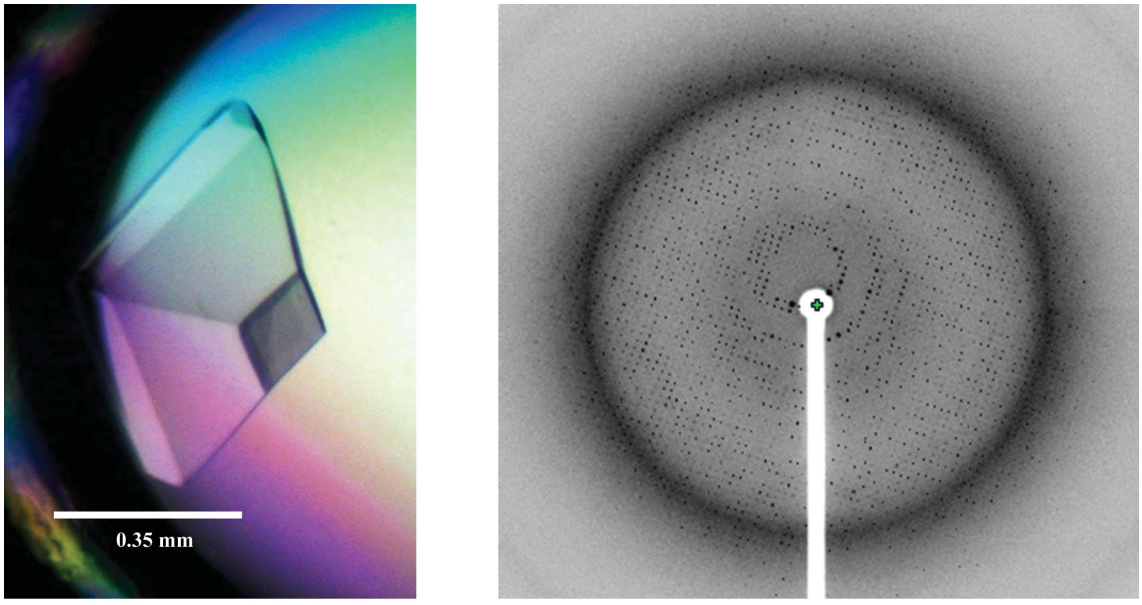


Figure 1
Superdex 200 16/60 gel-filtration analysis showing that Bxe_C0898 (blue peak) forms a stable tetramer of ∼140 kDa in solution. The red peaks show two of the four protein standards used for the calibration curve: peak I, conalbumin (75 kDa); peak II, ovalbumin (43 kDa). Inset, SDS–PAGE analysis of the column fractions, with the Bxe_C0898 monomer migrating at approximately 36 kDa (the expected molecular weight of the monomer is approximately 35 kDa).



(*a*) (*b*)

Figure 2
(*a*) Bxe_C0898 crystal grown in a sitting drop with 10% PEG 6000, 2% PEG 400, 250 m*M* CsCl buffered with 90 m*M* Tris pH 8.5 as the reservoir solution. (*b*) X-ray diffraction image of a Bxe_C0898 crystal collected to 2.5 Å resolution.

approximately 140 kDa (Fig. 1), consistent with the quaternary structure observed previously for LTTRs (Muraoka *et al.*, 2003; Smirnova *et al.*, 2004). Gel-filtration fractions were analyzed by SDS–PAGE and showed a predominant band at approximately 36 kDa as expected for the Bxe_C0898 monomer (34 999 Da). Streak-seeding produced high-quality crystals (Fig. 2) that diffracted to 2.5 Å resolution on Stanford Synchrotron Radiation Laboratory (SSRL) beamline 9-2. Processing of the data (Table 1) suggested that Bxe_C0898 crystallized with four molecules in the asymmetric unit of the tetragonal unit cell, with a solvent content of 61.19% and a Matthews coefficient of 3.17 $Å^3$ Da^{-1} (Matthews, 1968). Scaling and merging of the data resulted in an overall R_{merge} of 5.1% (49.4% in the highest resolution shell). Attempts to solve the structure by molecular replacement have thus far been unsuccessful. As a result, we are pursuing both a selenomethionine and a caesium phasing approach. We are complementing our structural characterization with DNA-protection assays in order to identify potential operator regions for this LTTR and to ultimately define its role in the regulation of the *box* pathway.

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