Biochemical and Biophysical Research Communications 423 (2012) 45–49

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Purification and crystallization of the ABC-type transport substrate-binding protein OppA from *Thermoanaerobacter tengcongensis*

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ARTICLE INFO

Article history:
Received 4 May 2012
Available online 22 May 2012

Keywords:
Characterization
Crystallization
OppA
Prokaryotic expression
Purification

ABSTRACT

Di- and oligopeptide- binding protein OppAs play important roles in solute and nutrient uptake, sporulation, biofilm formation, cell wall muropeptides recycling, peptide-dependent quorum-sensing responses, adherence to host cells, and a variety of other biological processes. Soluble OppA from *Thermoanaerobacter tengcongensis* was expressed in *Escherichia coli*. The protein was found to be >95% pure with SDS–PAGE after a series of purification steps and the purity was further verified by mass spectrometry. The protein was crystallized using the sitting-drop vapour-diffusion method with PEG 400 as the precipitant. Crystal diffraction extended to 2.25 Å. The crystal belonged to space group $C222_1$, with unit-cell parameters of $a = 69.395$, $b = 199.572$, $c = 131.673$ Å, and $\alpha = \beta = \gamma = 90°$.

1. Introduction

Bacteria can use peptides existing in their environment as a source of amino acids, carbon, nitrogen, and/or energy. Thus, the controlled transmembrane translocation of these solutes is very essential for their continued existence [1,2]. Living systems have evolved a variety of peptide uptake systems that can mediate the translocation of peptides across the bacterial cytoplasmic membrane. Among these, the largest and the most diverse transport system is the ATP-binding cassette (ABC) system [3–5]. The ABC system can be classified into two main classes: carbohydrate transporters and the di- and oligopeptide transporters [6,7]. All these transporters consist of five components: two homologous multi-transmembrane permeases (OppB and OppC) that constitute the hydrophobic translocator pore, two homologous intracellular nucleotide-binding domains (OppD and OppF) that support solutes transport by ATP hydrolysis, and one periplasmic substrate-binding protein (SBP), OppA, that determines the specificity of this system, binding and delivering the substrate to the permease domain selectively [2,8–11].

The substrate-binding proteins are unique in prokaryotic organisms in that they have differing amino acid sequences [3]. For Gram-negative bacteria, the SBPs are soluble proteins that reside in the periplasmic space, while for Gram-positive bacteria, they are either anchored to the surface of the cell membrane via N-terminal lipid modification or linked to the transmembrane domain covalently to prevent loss due to the lack of periplasmic space in Gram-positive bacteria [12–15].

The *Thermoanaerobacter tengcongensis* OppA orthologue $OppA_{TTE0054}$ belongs to a large subfamily of SBPs. Recently, there has been extensive investigation of the OppAs from many organisms. Beside the roles they play in solute and nutrient uptake, they are also involved in a variety of other physiological functions, including sporulation [16,17], biofilm formation [18], cell wall muropeptides recycling [19,20], peptide-dependent quorum-sensing responses [17,21], adherence to host cells [22,23] and even vaccine antigen development [24–26]. It has been reported that OppA- type SBPs contain an N-terminal bacterial-like signal peptide and a C-terminal hydrophobic domain with a long stretch of proceeding hydroxylated amino acids that are thought to be membrane anchored by a C-terminal membrane-spanning domain [6,27]. Structural studies have demonstrated that OppA consists of two domains connected by a hinge, and the rotation around the hinge determines the open or closed conformation [28,29]. The substrate-bound closed OppA can interact with the transmembrane domain for cargo deliverance, and the substrate-binding pocket is fairly large [30,31]. Early studies reported that OppA had a broad specificity and could bind 2–5 amino acid peptides

Abbreviations: SSRF, Shanghai synchrotron radiation facility; MAD, multiple-wavelength anomalous dispersion; MS, mass spectrometry.

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http://dx.doi.org/10.1016/j.bbrc.2012.05.067

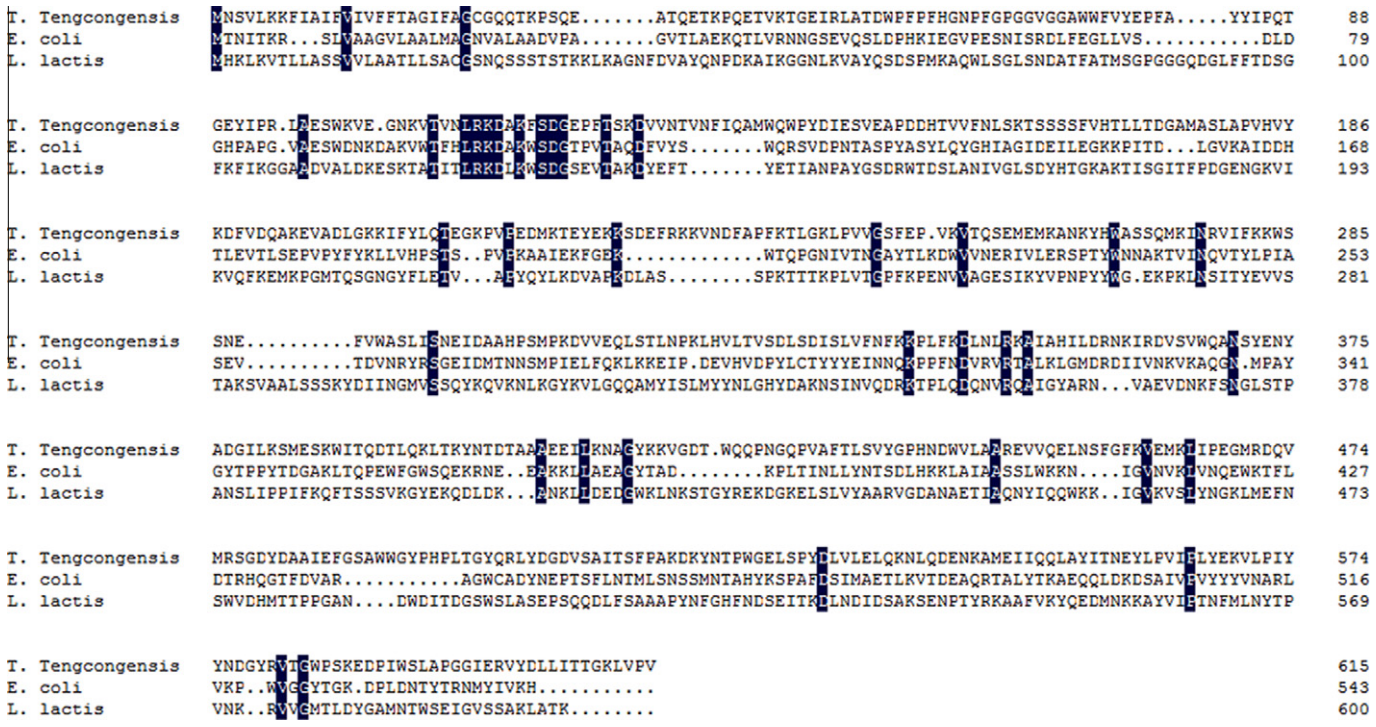

Fig. 1. Sequence alignment of OppA from *T. tengcongensis*, *E. coli*, and *L. lactis*. The black shadow indicates the identical amino acids among the three species.

regardless of their sequence, size and charge [15,32]. However, recent studies suggest that *E. coli* OppA has a preference for positively charged peptides that are 3- or 4-amino acids long, as the binding cleft generated by its domain I and domain II is negatively charged [33]. Additionally, *Lactococcus lactis* OppA tends to bind peptides that are 4–35 amino acids long and have at least one hydrophobic residue. Thus, the selectivity of the substrate-binding protein OppA appears more complicated than previously believed.

However, the sequence identity between OppA from *T. tengcongensis* and its orthologues from either *E. coli* or *L. lactis* (16.98% and 15.35%, respectively) (Fig. 1) is low.

In this study, we report the soluble expression of *T. tengcongensis* OppA orthologue ($OppA_{TTE0054}$) in *E. coli* and its subsequent purification and crystallization as the first step towards determining its three-dimensional structure.

2. Materials and methods

2.1. Materials

Enzymes used in the study for recombinant DNA techniques, such as Pfu polymerase, T4 DNA ligase, along with *Nde*I and *Xho*I restriction enzymes, were all purchased from New England Biolabs. All PCR buffers and dNTP mixtures were also obtained from New England Biolabs. Chromatographic columns, including a HisTrap HP 5-ml column, a Resource Q 1-ml column and a Superdex-200 16/300 column, were purchased from GE Healthcare. Crystal screening kits were purchased from Hampton Research.

2.2. Primer design and PCR amplification

Primers spanning amino acids 31–616 of OppA from *T. tengcongensis* were designed according to the published OppA sequence in GenBank (Accession No. NC_003869). To facilitate the subsequent cloning, *Nde*I and *Xho*I restriction endonuclease sites were attached to the upstream and downstream primers, respectively: forward, 5′-CGCCATATGCCGTCACAAGAGGCTACTCA-3′; reverse, 5′-CCCTCGAGTTATTTTACTGGGACTAGCTT-3′. The polymerase chain reaction was carried out using the genomic DNA of *T. tengcongensis* as a template. The sample was subjected to 32 cycles of denaturation (50 s, 94 °C), annealing (58 °C, 50 s), and extension (140 s, 68 °C) using a DNA Thermal Cycler (Eastwin).

The PCR product was separated on a 1% agarose gel, purified with a gel-extraction kit (DOUPSON) and digested with *Nde*I and *Xho*I overnight.

2.3. Cloning, expression and purification

The digested PCR product was cloned into the prokaryotic expression vector pET21b with *Nde*I and *Xho*I restriction enzyme sites, and the recombinant plasmid was transformed into *E. coli* strain BL21(DE3). Protein expression was induced by addition of 0.5 mM IPTG (final concentration) when the OD_{600} of the cell culture reached 0.6–0.8 in LB medium. The cells were allowed to grow for a further 12 h at 16 °C. The bacterial cells were harvested by centrifugation at 4000 rpm for 12 min at 4 °C. The cells pellets were resuspended in lysis buffer (50 mM Tris pH 7.7, 500 mM NaCl, 20 mM imidazole, 5% glycerol, 1 mM PMSF, 2 mM DTT) and homogenized by sonication on ice. The lysate was centrifuged at 15,000 rpm for 50 min at 4 °C to remove the cell debris.

All of the subsequent purification steps were performed at 4 °C. The purification procedures were performed using an AKTA-FPLC system (GE Healthcare). The clear supernatant was loaded onto a HisTrap HP 5-ml column that was initially equilibrated with buffer B (25 mM Tris pH 7.7, 500 mM NaCl, 500 mM imidazole, 2 mM DTT, 5% glycerol) and then subsequently equilibrated with buffer A (25 mM Tris pH 7.7, 500 mM NaCl, 20 mM imidazole, 2 mM DTT, 5% glycerol). The contaminant proteins were washed away from the column with 10 column volumes of 5% buffer B + 95% Buffer A. The target protein was eluted by 15% buffer B + 85% Buffer A. The fractions containing the target protein were pooled and transferred to buffer A (25 mM Tris pH 8.5, 80 mM NaCl, 2 mM DTT) using a HiPrep™ 26/10 desalting column. The desalted sample was applied onto a Resource Q column with buffer A (25 mM

Tris pH 8.5, 80 mM NaCl, 2 mM DTT) and buffer B (25 mM Tris pH 8.5, 500 mM NaCl, 2 mM DTT). The fractions containing the target protein were pooled and concentrated with a Vivaspin 30,000 molecular weight device (Sartorius Stedim Biotech) and further purified using a Superdex-200 10/300 gel filtration column using a buffer containing 10 mM HEPES (pH 7.4), 200 mM NaCl, 5 mM DTT and 5% glycerol. The purified protein was then analyzed by SDS–PAGE.

2.4. LTQ-ORBITRAP mass spectrometry analysis

The gel strips containing the target protein were removed from the SDS–PAGE gel, cut into small pieces and transferred into EP tubes as 1.8-mm diameter cores. The excised gel plugs were then digested with trypsin and sequentially washed with water and 100% ACN, each for 10 min. The ACN was changed twice to completely dehydrate the gel plugs. The gel plugs were dried in a SpeedVac, rehydrated with 1 mM triethyl ammonium bicarbonate (pH 8.3) and incubated with 1 μg of trypsin at 37 °C overnight. The gel plugs were further digested with trypsin after the addition of 20 μl of aqueous 1% ACN/1% FA to each gel plug followed by incubation at 37 °C for 1 h. The resulting peptide mixtures were analyzed with high-resolution nano-LC–MS on an Orbitrap (LTQ-ORBITRAP Velos, Thermo Fisher Scientific).

All data files were processed using Sequest and protein identification was performed by searching the *T. tengcongensis* database.

2.5. Crystallization

The purified protein was concentrated to approximately 40 mg ml^{-1}. The protein sample was centrifuged at 15,000 rpm for 30 min at 4 °C to clarify the solution prior to crystal screening trials. Initial screening was performed at 18 °C in 48-well plates using the sitting-drop vapor-diffusion method and sparse-matrix screen kits from Hampton Research (Crystal Screen I, Crystal Screen II, Crystal Screen Lite, Natrix, PEG/Ion, PEG/Ion2, PEGRx1, PEGRx2, Index), followed by refinements of the initial conditions with variations of pH, precipitant, and protein concentration. Typically, droplets consisting of 1.5 μl of protein solution and the equivalent volume of reservoir solution were equilibrated against 100 μl of reservoir solution.

2.6. X-ray crystallographic studies

X-ray diffraction data sets from the crystals were collected at the Shanghai Synchrotron Radiation Facility (SSRF) BL17U with a wavelength of 0.9762 Å on a MAR-CCD245 detector (Mar Research). A reservoir solution supplemented with 10% glycerol was used as a cryoprotectant. The crystal was immersed in the cryoprotectant solution for 5–10 s, collected with an appropriate nylon loop and then flash-cooled in a stream of liquid nitrogen at −173.15 °C (100 K). The exposure time was 1 s per frame, the detector distance was 150 mm and the oscillation range was 0.5° per frame. All intensity data were indexed, integrated, and scaled using the HKL2000 programs DENZO and SCALEPACK.

3. Results and discussions

3.1. Expression and purification of $OppA_{TTE0054}$

The full-length $OppA_{TTE0054}$ gene was cloned into the pET21b vector and OppA fused with a C-terminal 6× His tag was expressed in *E. coli*. Although the target protein could be purified using a buffer containing detergent Triton-X-100, the protein aggregated in the gel filtration column. Several detergents were tried to purify the target protein, but none of them could prevent aggregation of the target protein. The aggregation of the full-length protein may be due to the mis-folding of its N-terminal transmembrane signal peptide. Then a truncated protein without the N-terminal 30 amino acids was constructed and it was expressed as a soluble protein in *E. coli*. The truncated OppA could be purified without using detergents. After a series of purification steps (Table 1 and Fig. 2), the purified $OppA_{TTE0054}$ protein was >95% pure on SDS–PAGE gel stained with Coomassie brilliant blue (Fig. 3).

Table 1
Purification summary of $OppA_{TTE0054}$.

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Yield (%)
Soluble cell fraction	80	14.18	1134.55	100
HisTrap HP 5-ml	15	0.639	9.583	0.84
HiPrep™ 26/10	13	0.700	9.104	0.80
Resource Q	10	0.747	7.47	0.66
Superdex-200	6	0.74	4.44	0.39

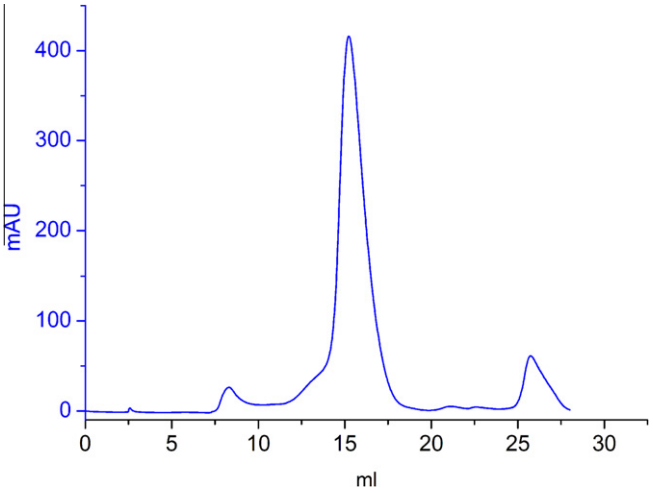

Fig. 2. The purification profile of $OppA_{TTE0054}$ on a Superdex-200.

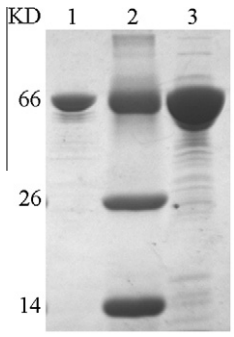

Fig. 3. SDS-PAGE of $OppA_{TTE0054}$. Lane 1, crystals; Lane 2, marker; Lane 3, the purified $OppA_{TTE0054}$ used for crystallization.

3.2. LTQ-ORBITRAP mass spectrometry analysis

The mass spectrometer (LTQ-Orbitrap) used in this study had a very high mass accuracy in the range of 10 ppm. The peptide mass fingerprinting (PMF) of the protein was observed and submitted to Sequest. Consequently, only the OppA protein from *T. tengcongensis* was credibly obtained by Sequest searching. The protein score was

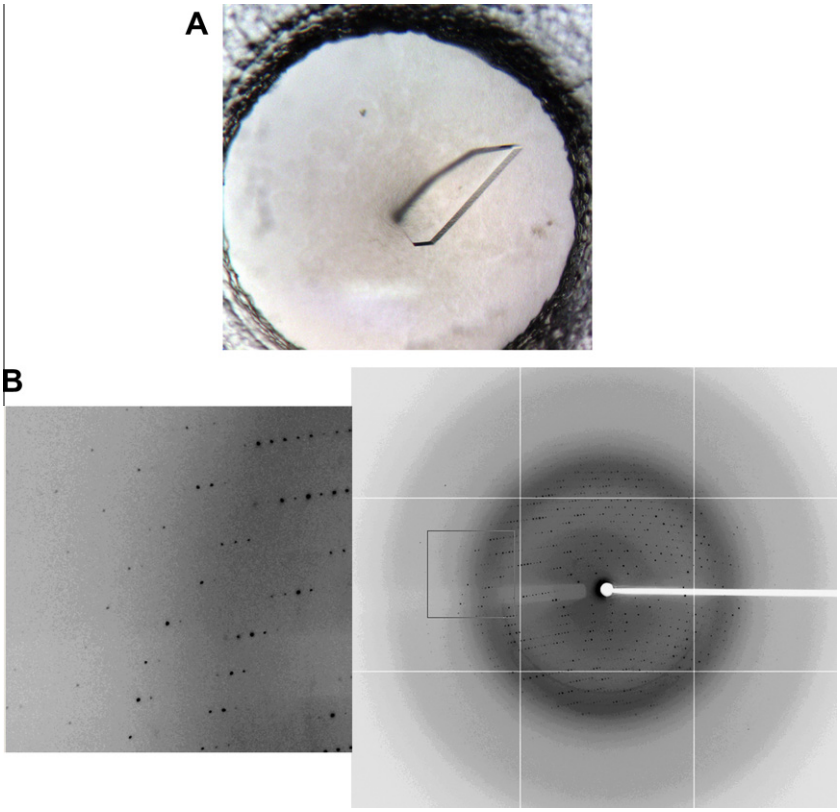

Fig. 4. (A) Typical crystal of $OppA_{TTE0054}$. (B) X-ray diffraction pattern from a $OppA_{TTE0054}$ crystal, the crystal diffracts to 2.25 Å.

1640.60 with a protein coverage of 74.68 (Table S1). LTQ-ORBITRAP MS analysis of the digested protein provided convincing evidence that this protein was OppA from *T. tengcongensis*.

3.3. Crystallization and data collection

Small crystals of $OppA_{TTE0054}$ appeared overnight and matured approximately two days later from one condition of a Crystal Screen II Kit containing PEG 400 as a precipitant. The crystallization condition was further optimized by varying the protein concentration, precipitant concentration, buffer pH, and additives. Larger crystals (Fig. 4a) were obtained using the sitting-drop rather than the hanging-drop vapor-diffusion method. The crystals were obtained at 18 °C by mixing 1.5 μl of protein with 1.5 μl of reservoir solution (0.1 M Cadmium chloride hydrate, 0.1 M Sodium acetate trihydrate pH 4.6, 30% v/v polyethylene glycol 400) and equilibrating against 100 μl of reservoir solution. The larger crystals were incubated with reservoir solution supplemented with 10% glycerol for X-ray diffraction.

An X-ray diffraction data set was collected from a single $OppA_{TTE0054}$ crystal to a maximum resolution of 2.25 Å (Fig. 4b). The crystal belonged to space group $C222_1$, with unit-cell parameters $a = 69.395$, $b = 199.572$, $c = 131.673$ Å (Table 2).

Although we obtained crystals and native X-ray diffraction data of $OppA_{TTE0054}$, we were unable to determine its structure via a molecular replacement method because of the lack of a suitable search model. The multiple-wave-length anomalous dispersion (MAD) method will be used to solve the structure as there are 12 methionine residues in one $OppA_{TTE0054}$ molecule. Now, we have already obtained crystals from a seleno-methionine derivative protein and structure determination is currently in progress.

Table 2
Data collection and processing statistics.

Space group	$C222_1$
Unit cell parameters (Å)	$a = 69.395$, $b = 199.572$, $c = 131.673$, $\alpha = 90°$, $\beta = 90°$, $\Gamma = 90°$
Wavelength (Å)	0.9762
Resolution (Å)	30–2.25
No. reflections (observed)	522446
No. reflections (unique)	43616
Completeness (%)	99.4 (99.3)
$\langle I/\sigma(I)\rangle$	35.28 (3.36)
Rsym (%)	8.6 (51.3)

Acknowledgments

This work was supported by the Ministry of Science and Technology (2011CB910500 and 2012CB911100) and National Natural Science Foundation (31030020 and 31170679). We would like to thank the staff at the SSRF BL17U beamline for their assistance in data collection and Di Miao for her help with MS data analysis. We are also grateful to Prof. Maojun Yang of Tsinghua University and Shentao Li of Capital Medical University for their technical assistance with data processing, valuable comments and critical discussion.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.067.

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