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Cloning, expression, purification and preliminary X-ray analysis of EstN2, a novel archaeal α/β -hydrolase from *Candidatus Nitrososphaera gargensis*

EstN2 is a novel α/β -hydrolase originating from the ammonia-oxidizing thaumarchaeon *Candidatus Nitrososphaera gargensis*. The genome of the organism was sequenced and genes conferring putative lipolytic activity were amplified and cloned into *Escherichia coli* as a heterologous host. Through function-based screening, esterase and lipase activity was detected. A recombinant enzyme designated EstN2 was successfully expressed, purified and crystallized. The crystals belonged to space group *I*2, with one molecule per asymmetric unit, and diffracted X-rays to 1.5 Å resolution.

1. Introduction

Candidatus Nitrososphaera gargensis (the '*Candidatus*' designation is omitted in the following text) is one of the few characterized members of the novel third archaeal phylum thaumarchaeota (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010). This moderately thermophilic organism was originally obtained from microbial mats of a pond associated with a terrestrial hot spring (Garga spring; 318 K) located in the northeastern part of the Baikal rift zone (Buryat Republic, Russia). The chemolithoautotrophic nitrifying archaeon was enriched and its genome was analyzed. This analysis revealed that the organism is equipped with a subset of conserved enzymes necessary for archaeal ammonia oxidation to nitrite, as well as components that enable it to use urea and possibly cyanate as alternative nitrogen sources. Interestingly, analysis of the genome indicated parts that may have been obtained by lateral gene transfer from bacteria and euryarchaeota and contribute to a versatile energy and carbon metabolism and a high metabolic flexibility (Spang *et al.*, 2012). *N. gargensis* is able to perform CO₂ fixation, presumably through a modified 3-hydroxypropionate/4-hydroxybutyrate carbon-fixation pathway. Considering the carbon metabolism, hexose degradation by the oxidative pentose phosphate pathway cannot be excluded, but the non-oxidative pentose phosphate pathway seems to be predominant. All of the genes encoding gluconeogenic enzymes involved in the synthesis of fructose 6-phosphate are present within the genome. Lipid side chains are produced in the mevalonate pathway. The cell membrane of *N. gargensis* mostly consists of crenarchaeol, a glycerol dialkyl glycerol tetraether (GDGT) with a unique cyclohexane moiety, *i.e.* the novel core membrane lipid GDGT-X and its regioisomer (Pitcher *et al.*, 2010). A repertoire of genes encodes transporters for the cellular uptake of sugars, organic acids, amino acids, organics, sulfonates, oligopeptides and glycerol.

The significance of the α/β -hydrolase EstN2 for the metabolism of cellular compounds of *N. gargensis* is still unclear and needs to be elucidated. An involvement in the conversion of the incorporated organic molecules in some of the abovementioned metabolic steps as well as in cell-membrane modifications is possible, and the findings will deliver new insights into the interesting physiology of this archaeon. Biochemical and structural investigations of this enzyme will help to solve the *in vivo* cellular function of EstN2.

2. Materials and methods

2.1. Macromolecule production

Biomass samples from the mats were enriched over more than six years in mineral medium containing different ammonia concentrations (Lebedeva *et al.*, 2005; Hatzepichler *et al.*, 2008). *N. gargensis* was identified within enriched cultures by molecular methods and was further cultivated until cell harvest for the isolation of genomic DNA and subsequent genome sequencing and annotation (Spang *et al.*, 2012). Six different genes annotated as α/β -hydrolase fold proteins conferring putative lipolytic activity were amplified from the genomic DNA by PCR, among them the gene *estN2* (Ngar_c30910, putative α/β -hydrolase fold protein, start 2422483, end 2421650). The PCR product generated with the primers EstN2_for (5'-CTCTACGG-GATAGAAGATATTAAAC-3') and EstN2_rev (5'-TTGCCATTATAGCAAACTACTAC-3') with a size of 0.8 kb was ligated into pDrive cloning vector (3.85 kb; Qiagen, Hilden, Germany). The subsequent plasmid was transformed into *Escherichia coli* XL1-Blue competent cells by heat shock and tested for lipolytic activity on LB (Luria-Bertani) medium agar plates containing 1% (v/v) tributyrin. Active clones, which express carboxylesterases, produce a clearing zone around the colony after a certain time of incubation. The clone expressing EstN2 reproducibly showed a clear halo after 18 h of incubation at 310 and 319 K (up to 48 h); thus, the 840 bp gene *estN2* was cloned into pET-21a (Novagen, Darmstadt, Germany) with primers containing restriction sites for directed cloning into the multiple cloning site. The primers were also designed for in-frame translation of a C-terminal His₆ tag encoded by the vector sequence that allows affinity purification of the respective protein [EstN2_NdeI_for (5'-CATATGCCTTTTCTATCCTCGCCTTC-3') and EstN2_HindIII_rev (5'-AAGCTTCCCAACAGGCTTTACATCCTTTGTC-3'); restriction sites are underlined]. The construct was verified by sequencing.

The plasmid for EstN2 was transformed into *E. coli* Rosetta-gami 2 (DE3) competent host cells (Novagen, Darmstadt, Germany) by heat shock. The cell strain contained the chloramphenicol-resistant pRARE2 plasmid that supplied seven rare tRNAs and additionally conferred enhanced disulfide-bond formation onto the recombinantly produced proteins. *E. coli* pre-cultures of the strain harbouring the plasmids were grown overnight on Luria-Bertani (LB) medium at 310 K and used as a 1% (v/v) inoculum for 1 l LB expression cultures containing 100 $\mu\text{g ml}^{-1}$ ampicillin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol. The cultures were incubated at 301 K until an OD₆₀₀ value of 0.8 was reached and were then induced by the addition of 1 mM IPTG (isopropyl β -D-1-thiogalactopyranoside). After an additional incubation period at 301 K for 18 h, the cells were harvested by centrifugation. The cell pellet was stored at 253 K until further use.

For Ni-NTA affinity purification of EstN2_His₆, the cells were thawed on ice and resuspended in 8 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole pH 8.0). The cells were disrupted in two steps using a French pressure cell and subsequent sonication on ice (2 $\times$ 5 min with 5 min interruption; ultrasonication processor UP 200S, 24 kHz, 200 W, Dr Hielscher GmbH, Teltow, Germany). Cell debris was removed from the crude cell extract by centrifugation at 13 000 rev min⁻¹ for 30 min at 277 K (Sorvall RC6+ centrifuge, SS-34 rotor; Thermo Scientific, Braunschweig, Germany). The crude extract was incubated on ice with 1 ml Ni-NTA resin (Ni-NTA coupled to Sepharose; Qiagen, Hilden, Germany) per 4 ml of lysate on a platform shaker (50 rev min⁻¹) for 3 h. The sample was loaded onto a polypropylene column, the flowthrough was collected for analysis and nonspecifically bound proteins were removed by washing twice with one column volume (CV; 4 ml) of wash buffer (50 mM NaH₂PO₄,

Table 1

Macromolecule-production information.

Source organism	<i>N. gargensis</i>
DNA source	<i>N. gargensis</i>
Forward primer	CATATGCCTTTTCTATCCTCGCCTTC
Reverse primer	AAGCTTCCCAACAGGCTTTACATCCTTTGTC
Cloning vector	pDrive
Expression vector	pET-21a
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	MPFLSSPSNSVSTYYEDHGDARSYPLVLIHPGNNLIWDYE-IQLLLKSGFRVIAVELRGHHRTNMGKTGAYTMQDLIDDLRLLEHLNIGKCTTIIGHSGGISSMYAAQHPGVDAIIMI-NGSPKKFQEKDLEKHFRTREVAITQGMKALAEHLVSLDE-ARLDFADKRHADFFREVTFTSVGEFVAATVALYTIIPGNV-VQGLRASGCKVFAIVGSDDDVFMRLIKETKEEIPEMELRV-LQGSDDHWVIEKPKEMYDILMGFLAIVTKDVKPVGKLAAL-LEHHHHHH

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	iQ plate (TTP Labtech)
Temperature (K)	292
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	50 mM NaCl, 50 mM HEPES pH 7.5
Composition of reservoir solution	0.2 M magnesium acetate, 20% PEG 3350
Volume and ratio of drop	600 nl, 1:1
Volume of reservoir (μl)	50

300 mM NaCl, 20 mM imidazole pH 8.0). Elution of the His₆-tagged protein followed the addition of 6 $\times$ 0.5 ml elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole pH 8.0) to the resin. The Ni-NTA purification fractions were analyzed on 12% SDS-PAGE gels stained with Coomassie Brilliant Blue and protein concentrations were measured using the Bradford method (Roti-Quant, Carl Roth, Karlsruhe, Germany). Fractions that contained a clear protein band on SDS-PAGE with a size according to EstN2_His₆ were combined in a Vivaspin column [5000 molecular-weight cutoff (MWCO), Sartorius, Göttingen, Germany] and concentrated by centrifugation (Centrifuge 5804R, Eppendorf, Hamburg, Germany) at 277 K and 5000 rev min⁻¹ to a volume of 1 ml. The protein was washed twice to remove imidazole by the addition of 4 ml HEPES buffer (50 mM NaCl, 50 mM HEPES pH 7.5) and subsequent centrifugation to a final volume of 0.3 ml.

The average yield of pure sample was 20 mg from 1 l of culture. The enzyme EstN2_His₆ with the noncleavable His₆ tag has a size of 290 amino acids and a molecular mass of 32 610.1 Da. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Initial crystallization experiments were carried out with six commercial crystallization screens (The Classics, Classics II, PEGs, PEGs II, pHClear and pHClear II Suites from Qiagen) at the EMBL Hamburg high-throughput crystallization facility (Mueller-Dieckmann, 2006). All initial screens were performed using a protein concentration of 10 mg ml⁻¹ and the sitting-drop vapour-diffusion method at 292 K in 96-well iQ plates (TTP Labtech, Melbourn Science Park, Royston, England). 300 nl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 50 μl reservoir solution. The initial screens resulted in six lead conditions. Four of these crystals diffracted X-rays to better than 2 Å resolution without further optimization. All of these crystals belonged to the same space group, with very similar unit-cell parameters. The best crystals grew in a drop consisting of 0.2 M magnesium acetate, 20% PEG 3350 (The PEGs Suite, solution No. 73). The

corresponding crystals diffracted X-rays to 1.5 Å resolution. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Prior to data collection, a single EstN2_His₆ crystal (Fig. 1) was soaked for about 5 s in mother liquor augmented with 15% glycerol as a cryoprotectant. The crystal was then flash-cooled to 77 K in liquid nitrogen. X-ray diffraction data were collected on beamline P14 of PETRA III, EMBL Hamburg, Germany at 100 K using a PILATUS 6M detector. A total of 360 images were acquired using an oscillation range of 0.5°. The data were indexed and integrated using XDS (Kabsch, 2010) and scaled with SCALA (Winn *et al.*, 2011). Table 3 summarizes the data-collection and processing statistics.

3. Results and discussion

A novel α/β -hydrolase from the ammonia-oxidizing archaeon *N. gargensis* was cloned and overexpressed in *E. coli* Rosetta-gami 2 (DE3). It showed reproducible carboxylase activity on tributyrin agar plates and on 4-nitrophenyl substrates with different carbon-chain residues (J. Chow, to be published).

A sequence alignment of EstN2 against the NCBI database using BLASTX 2.2.29 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; Altschul *et al.*, 1997) revealed the highest similarity to a putative hydrolase/acyltransferase of the α/β superfamily from the related *Candidatus* Nitrososphaera evergladensis SR1 (NCBI accession No. AIF85481.1; 91% query coverage, *E*-value 2×10^{-129} , 70% identity). The biochemical properties of this putative enzyme have not been studied to our knowledge (Zhalnina *et al.*, 2014). Furthermore, the BLASTX alignment (date of search: 5 August 2014) resulted in a hypothetical protein from the bacterium JKG1 (NCBI accession No. WP_029214597.1; 89% query coverage, *E*-value 1×10^{-18} , 70% identity), an α/β -hydrolase from the crenarchaeote *Sulfolobus islandicus* (NCBI accession No. WP_014514106.1; 91% query coverage, *E*-value 5×10^{-18} , 31% identity) and a 3-oxoadipate enol-lactonase from the alphaproteobacterium *Roseibium* sp. TrichSKD4 (NCBI accession No. WP_009468162.1; 89% query coverage, *E*-value 5×10^{-18} , 28% identity). Lactonase activity could not be detected on *N*-acyl homoserine lactones containing different carbon-chain residues from C4 to C12 in combination with the reporter strain *Agrobacterium tumefaciens* NTL4. Additionally, in pyocyanin assays using *Pseudomonas fluorescence* PA01 (Gallagher *et al.*, 2002), as well as in cross-streak experiments with autoinducer-producing strains such as *Janthinobacterium* sp. HH01 and *Sinorhizobium fredii* NGR234, EstN2_His₆ did not show any visible influence (J. Chow, to be published). Thus, the function of EstN2 within its original organism may not be related to quorum-sensing or quorum-quenching mechanisms and remains to be further elucidated.

The recombinant protein was purified using Ni-NTA affinity chromatography and resulted in pure protein according to SDS-PAGE analysis. Since phosphate buffers often result in undesired salt crystals in crystallization experiments, a buffer exchange was performed using a Vivaspin concentrator. The final sample that was subjected to crystallization contained protein at a concentration of 10 mg ml⁻¹ in a buffer consisting of 50 mM NaCl, 50 mM HEPES pH 7.5. The initial crystallization experiments were performed using commercial screens at the EMBL Hamburg high-throughput crystallization facility (Mueller-Dieckmann, 2006) and resulted in several good-quality crystals. The best data were collected from a crystal that diffracted to 1.5 Å resolution. This crystal was obtained from conditions consisting of 0.2 M magnesium acetate, 20% PEG 3350. A

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	MX-P14, PETRA III
Wavelength (Å)	0.976
Temperature (K)	100
Detector	PILATUS 6M
Crystal-to-detector distance (mm)	265
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	0.5
Space group	<i>I</i> 2
Unit-cell parameters (Å, °)	<i>a</i> = 50.95, <i>b</i> = 65.46, <i>c</i> = 84.59, $\alpha = \gamma = 90$, $\beta = 104.7$
Mosaicity (°)	0.152
Resolution range (Å)	51–1.50 (1.58–1.50)
Total No. of reflections	142183 (20953)
No. of unique reflections	42828 (6251)
Completeness (%)	99.3 (99.3)
Multiplicity	3.3 (3.4)
$\langle I/\sigma(I) \rangle$	21.2 (7.3)
<i>R</i> _{merge} (%)	3.3 (17.0)
<i>R</i> _{p.i.m.} (%)	2.1 (10.9)
Overall <i>B</i> factor from Wilson plot (Å ²)	12.1

data set of 360 images was collected on PETRA III beamline P14 at EMBL Hamburg, Germany. The crystal belonged to space group *I*2, with unit-cell parameters *a* = 50.95, *b* = 65.46, *c* = 84.59 Å, $\alpha = \gamma = 90$, $\beta = 104.7^\circ$. Based on the calculated Matthews coefficient, the asymmetric unit is expected to contain one molecule, with a solvent content of 44% (Matthews, 1968). Initial phases were calculated using the automated molecular-replacement program MOLREP v.11.0 (Vagin & Teplyakov, 2010) as implemented in CCP4 v.6.3.0 (Winn *et al.*, 2011) and the structure of *Streptomyces lividans* chloroperoxidase L (PDB entry 1a88; Hofmann *et al.*, 1998) as a search model. Owing to the low sequence identity (24%) between the two proteins, the electron-density map from the initial phasing was of poor quality, with a current *R* factor of 0.473. Small fragments of the core region could be built into the density map. We will continue to further improve the quality of this model through iterative cycles of manual model building and subsequent refinement with Coot v.0.7 (Emsley *et al.*, 2010) and REFMAC5 (Murshudov *et al.*, 2011), respectively.

Figure 1

Crystals of EstN2_His₆ from *N. gargensis*. The prism-shaped crystals shown here are about 175 × 100 × 50 μm in size.

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