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Purification, crystallization and preliminary X-ray crystallographic analysis of the UDP-*N*-acetylmuramoyl-tripeptide-*D*-alanyl-*D*-alanine ligase (MurF) from *Acinetobacter baumannii*

The emergence and global spread of multidrug-resistant *Acinetobacter baumannii* strains are major threats to public health. Inhibition of peptidoglycan biosynthesis is an effective strategy for the development of antibiotics. The ATP-dependent UDP-*N*-acetylmuramoyl-tripeptide-*D*-alanyl-*D*-alanine ligase (MurF) that is responsible for the last step of peptidoglycan biosynthesis is a validated target for the development of antibiotics. Crystals of *A. baumannii* MurF in complex with ATP were grown by the microbatch crystallization method at 295 K. The crystals belonged to space group *P*322₁, with unit-cell parameters *a* = *b* = 85.42, *c* = 129.86 Å. Assuming the presence of one molecule in the asymmetric unit, the solvent content was estimated to be about 54.32%.

1. Introduction

Peptidoglycan (or murein), which forms bacterial cell walls, is a thin sheet composed of *N*-acetylglucosamine (GlcNAc), *N*-acetylmuramic acid (MurNAc) and short peptides consisting of L-alanine, D-alanine, D-glutamic acid and either L-lysine or *meso*-diaminopimelic acid (*m*-DAP). The two alternating sugar derivatives linked by β-1,4-glycosidic bonds are the repeating units of peptidoglycan. L-Alanine, D-glutamic acid, *meso*-diaminopimelic acid (or L-lysine) and D-alanine-D-alanine are successively attached to the MurNAc moiety by four ADP-forming ligases: MurC, MurD, MurE and MurF, respectively (Barreateau *et al.*, 2008). The linear glycan chains formed by the sugar repeats are cross-linked with each other through bonding between the short peptides, forming a mesh-like rigid layer.

UDP-*N*-acetylmuramyl-pentapeptide [UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP(or L-Lys)-D-Ala-D-Ala] is the monomeric precursor of peptidoglycan and is synthesized from fructose 6-phosphate by successive enzyme reactions in the cytosol. MurF is the enzyme responsible for the last step in the biosynthesis of UDP-*N*-acetylmuramyl-pentapeptide. MurF catalyzes the addition of D-Ala-D-Ala dipeptide to UDP-*N*-acetylmuramyl-tripeptide [UDPMurNAc-L-Ala-γ-D-Glu-*m*-DAP(or L-Lys)] in an ATP-dependent manner (Duncan *et al.*, 1990; Anderson *et al.*, 1996). The γ-phosphate of ATP is transferred to the C-terminal carboxylate of UDP-*N*-acetylmuramyl-tripeptide to form an acyl phosphate intermediate, which is subsequently attacked by the amino group of the incoming dipeptide to form a peptide bond in a non-ribosomal fashion (Falk *et al.*, 1996; Vaganay *et al.*, 1996).

Acinetobacter baumannii is a difficult-to-treat pathogen that is responsible for serious nosocomial infections accompanied by high mortality, prolonged hospital stay and increased medical costs (Cisneros *et al.*, 1996; Peleg *et al.*, 2008; Seifert *et al.*, 1995). The emergence and global spread of multidrug-resistant *A. baumannii* strains are great threats to public health (Taccone *et al.*, 2006; Valencia *et al.*, 2009) and thus the development of novel approaches for treating infections caused by *A. baumannii* is urgently required. Enzymes involved in the synthesis of peptidoglycan are attractive targets for pharmaceutical investigation because they are essential for bacterial survival and their counterparts are not found in human hosts (Bugg & Walsh, 1992). From this perspective, MurF is a validated target for antibacterial drug discovery. Several inhibitors have

Table 1
Macromolecule production.

Source organism	<i>A. baumannii</i>
DNA source	Synthetic DNA
Forward primer	5'-ATATACATATGCATCATCACCATCATCATGCATCACTT-ATCAGATGCATCACTTCAACC-3'
Reverse primer	5'-CGGATCAAGCTTTTAAAGTTTCTCCATCAATCAGC-3'
Cloning vector	pUC57
Expression vector	pRSET-A
Expression host	<i>E. coli</i> strain B834(DE3)
Complete amino-acid sequence of the construct produced†	MHHHHHMTSTTSTVPLEPWAQQLQQATQGYWHKQIPQ-TEIKRILTDSRHAESGDAFLALKGERFDAHNFVAQVVG- GCQVAIVERPIDAEIAQLVVADTRLALGQLGAYRREQNA- QLKVIALTGSSGKTTTKEMLGSILSRLAPTILITRGNLNN- DLGVPMMLELRKEHQYAVMELGANHQGEIDYTSKIVQP- HVAGILNIGTAHLGEFGGRDGCRAKSEIYRHILPQGV- IVPQQDDFTAEIREAAKSHQIMSGEGGVFATEIELLP- QSANFQLHTPQGSFVRLPFAGEHNQVQATAAVAFALAL- GVSLEDIVKLEQAQGAQGRNLNFIQKAPHLFIDDTYNAN- PTSMRAAAQVLLQQNGIKVMVMDIGELGDSSWQEHHD- GRDLAELPLDHIVAVGQFASAALEGAGLHSTKLKAFQTQ- AEALPFLINLIQTHQPQMSFLFKGSRFTHMETLMADLM- EKL

† In the amino-acid sequence, M represents selenomethionine.

been developed against the MurF proteins from pathogenic bacteria including *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* (Longenecker *et al.*, 2005; Hrast *et al.*, 2013). To date, crystal structures of *E. coli* MurF, *Thermotoga maritima* MurF and *S. pneumoniae* MurF, which have sequence identities to *A. baumannii* MurF of 41, 32 and 29%, respectively, have been reported (Yan *et al.*, 2000; Longenecker *et al.*, 2005; Favini-Stabile *et al.*, 2013; Hrast *et al.*, 2013). Here, we report the overexpression, crystallization and preliminary X-ray crystallographic analysis of the *A. baumannii* MurF (*AbMurF*)–ATP complex as a first step towards structure determination.

2. Materials and methods

2.1. Cloning, expression and purification

We found the gene for *A. baumannii* UDP-*N*-acetylmuramoyl-tripeptide-*D*-alanyl-*D*-alanine ligase (*AbMurF*) by searching the GenBank sequence database with the gene name, and confirmed the

Figure 1
Purified *AbMurF* on 12% SDS–PAGE. Lane *M* contains molecular-weight size markers (labelled in kDa).**Table 2**
Crystallization.

Method	Microbatch crystallization method
Plate type	72-well HLA plates
Temperature (K)	295
Protein concentration (mg ml ⁻¹)	15
Buffer composition of protein solution	150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM DTT
Composition of reservoir solution	0.1 M sodium citrate–citric acid pH 5.5, 20% PEG 3000
Volume and ratio of drop	2 µl, 1:1

gene (accession No. CP001172) by using a homology search with other bacterial MurFs. The full-length gene (1422 base pairs) with additional sequences corresponding to the start codon and six histidines at the N-terminus was synthesized by a commercial gene-synthesis service (Cosmogenetech, Seoul, Republic of Korea). The entire *AbmurF* gene product was inserted downstream of the T7 promoter of the expression plasmid pRSET-A (Invitrogen, Carlsbad, California, USA) using *NdeI* and *HindIII* (Table 1). After verifying the DNA sequence, the plasmid DNA was transformed into the *E. coli met⁻* auxotrophic strain B834(DE3) (Novagen). To produce selenomethionine-labelled *AbMurF*, the transformed cells were grown in M9 media containing 25 mg l⁻¹ selenomethionine (Acros Organics), 100 µg ml⁻¹ ampicillin (Duchefa) and 30 µg ml⁻¹ chloramphenicol (Duchefa) at 310 K to an OD₆₀₀ of ~0.5 and expression was induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (Duchefa). After 16 h induction at 293 K, the cells were harvested and resuspended in 300 mM NaCl, 25 mM Tris–HCl pH 7.4, 10% glycerol, 0.1% Tween 20. After sonication and centrifugation, the six-histidine-tagged *AbMurF* in the supernatant was sequentially purified using a nickel–nitrilotriacetic acid column (Qiagen) with a washing buffer consisting of 300 mM NaCl, 25 mM Tris–HCl pH 7.4, 10% glycerol, 20 mM imidazole and an elution buffer consisting of 300 mM NaCl, 25 mM Tris–HCl pH 7.4, 10% glycerol, 250 mM imidazole and a Superdex 200 HR 16/60 size-exclusion column (GE Healthcare) with buffer consisting of 150 mM NaCl, 20 mM HEPES pH 7.4, 1 mM dithiothreitol (DTT). The *AbMurF* protein eluted with an apparent molecular weight in the region of 50 kDa. The purified recombinant *AbMurF* protein was concentrated to ~15 mg ml⁻¹ for crystallization (Fig. 1).

2.2. Crystallization

Crystallization experiments were performed in the presence of ATP by the microbatch crystallization method at 295 K (Table 2).

Figure 2
Crystals of MurF from *A. baumannii*.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

	Peak	Inflection	Remote
Diffraction source	5C, Pohang Light Source		
Wavelength (Å)	0.97919	0.97934	0.97151
Temperature (K)	100	100	100
Detector	ADSC Q315r	ADSC Q315r	ADSC Q315r
Crystal-to-detector distance (mm)	270	270	270
Rotation range per image (°)	1	1	1
Total rotation range (°)	200	200	200
Exposure time per image (s)	0.5	0.5	0.5
Space group	P32 ₁		
Unit-cell parameters (Å)	$a = b = 85.42$, $c = 129.86$		
Mosaicity (°)	0.472	0.480	0.525
Resolution range (Å)	50.00–1.90 (1.93–1.90)	50.00–1.90 (1.93–1.90)	50.00–1.90 (1.93–1.90)
Total No. of reflections	1140872	1146829	984609
No. of unique reflections	43695 (2097)	43626 (2120)	43685 (2096)
Completeness (%)	99.3 (98.5)	99.3 (98.6)	99.1 (97.8)
Multiplicity	9.3 (6.9)	9.2 (6.8)	7.7 (5.7)
$\langle I/\sigma(I) \rangle$	43.8 (6.3)	42.6 (5.7)	38.3 (5.0)
$R_{\text{meas}}^{\dagger}$ (%)	8.7 (30.0)	7.8 (30.8)	7.7 (31.6)
Overall B factor from Wilson plot (Å ²)	18.2	18.8	19.3

$\dagger R_{\text{meas}}$ is estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where N is the data multiplicity.

Small drops composed of 1 μ l protein solution containing 1.5 mM ATP and an equal volume of crystallization reagents were pipetted under a layer of a 1:1 mixture of silicone oil and paraffin oil in 72-well HLA plates (Nunc). Screening for crystallization conditions was performed with all available screening kits from Hampton Research, Axygen Biosciences, Emerald Bio and Microlytic. Initial crystals (Fig. 2) were grown in a precipitant solution consisting of 0.1 M sodium citrate–citric acid pH 5.5, 20% polyethylene glycol (PEG) 3000 (condition No. 43 of MCSG I from Microlytic).

2.3. Data collection and processing

Since the initial crystals were large enough for data collection, we used them without further optimization. For data collection, crystals were mounted using a 100 μ m MicroMount polymer loop (MiTeGen, Ithaca, New York, USA) and were cooled to 100 K using a Cryostream cooler. Before mounting, crystals were briefly immersed in the mother liquor containing 20% (w/v) glycerol as a cryoprotectant. A 1.90 Å resolution MAD data set was collected at three wavelengths (peak, 0.97919 Å; inflection, 0.97934 Å; remote, 0.97151 Å) using an ADSC Q315r CCD on beamline 5C of the Pohang Light Source (Pohang, Republic of Korea). The exposure time to the synchrotron radiation was 0.5 s. For each data set, 200 diffraction images with a 1° oscillation width were collected with the crystal-to-detector distance set to 270 mm. Diffraction data were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL-2000* program suite (Otwinowski & Minor, 1997). The data-collection statistics are shown in Table 3.

3. Results and discussion

The crystals belonged to the primitive trigonal space group *P*32₁, with unit-cell parameters $a = b = 85.42$, $c = 129.86$ Å. The crystal volume per unit molecular weight (V_M ; Matthews, 1968) was about

2.69 Å³ Da^{−1}, with a solvent content of 54.32% by volume, when the asymmetric unit was assumed to contain one molecule (Table 3). Experimental phasing was performed using the *AutoSol* program (Terwilliger *et al.*, 2009) in the *PHENIX* suite (Adams *et al.*, 2010). The *AutoSol* program identified 14 selenium sites and produced a phase set with a figure of merit of 0.66. The experimental electron-density map clearly revealed an ATP molecule bound to *Ab*MurF. Model building is now in progress. Despite several structural studies on MurF from other microorganisms (Yan *et al.*, 2000; Longenecker *et al.*, 2005; Favini-Stabile *et al.*, 2013; Hrast *et al.*, 2013), there has been no structural information reported on the MurF–ATP complex. Since MurF is an ATP-dependent enzyme, the crystal structure of the *Ab*MurF–ATP complex will provide insights into the reaction mechanism of MurF.

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