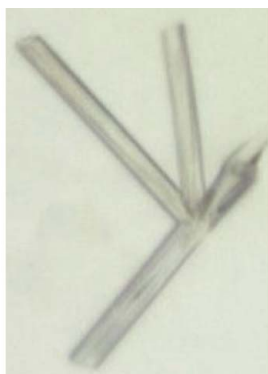


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Purification, crystallization and data collection of methicillin-resistant *Staphylococcus aureus* Sar2676, a pantothenate synthetase

Sar2676, a pantothenate synthetase with a molecular weight of 31 419 Da from methicillin-resistant *Staphylococcus aureus*, has been expressed, purified and crystallized at 293 K. The protein crystallizes in a primitive triclinic lattice, with unit-cell parameters $a = 45.3$, $b = 60.5$, $c = 117.6$ Å, $\alpha = 87.2$, $\beta = 81.2$, $\gamma = 68.4^\circ$. A complete data set has been collected to 2.3 Å resolution at the ESRF. Consideration of the likely solvent content suggested the asymmetric unit to contain four molecules. This has been confirmed by molecular-replacement phasing calculations, which give a solution with four monomers using a monomer of pantothenate synthetase from *Escherichia coli* (PDB code 1iho), which is 41% identical to Sar2676, as a search model.

1. Introduction

The widespread use of antimicrobials is contributing to a significant rise in drug-resistant bacteria. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of the *S. aureus* bacterium that exhibits broad-spectrum resistance to a variety of clinically relevant antibiotics (Foster, 1996). There are now 16 known epidemic strains of MRSA, two of which (Nos. 15 and 16) are believed to be particularly transmissible (Johnson *et al.*, 2001). Genes expressing antibiotic resistance in *S. aureus* can either be encoded in the chromosome or in a plasmid. The resistance genes in MRSA are integrated into the chromosome and thus their diffusion into other bacteria is more limited. Although this is reassuring, MRSA itself has now become a significant healthcare issue, spreading from intensive-care units throughout hospitals and now into the wider community. These community-acquired infections are viewed with particular unease (Kuehnert *et al.*, 2005; Styers *et al.*, 2006; Boyle-Vavra & Daum, 2007) as over 2 billion people worldwide carry some form of *S. aureus*. Of these 2 billion, it is anticipated as many as 53 million (2.7% of carriers) carry MRSA (Paul, 2006). If this MRSA begins to dominate *S. aureus* carriers, then the acute lack of novel and effective antibiotics could become a very serious matter.

Pantothenate (vitamin B₅) is a key precursor of the 4-phosphopantetheine moiety of coenzyme A (CoA) and the acyl carrier protein (ACP). Both are essential cofactors for cell growth and are involved in essential biosynthetic pathways (Jackowski, 1996). Microorganisms and plants synthesize pantothenate, while animals obtain this nutrient from their diet (Maas, 1960). The pantothenate-biosynthetic pathway offers a potential target for the design of new drugs against microbial pathogens. The full biosynthetic pathway to pantothenate comprises four enzymes and is best understood in *Escherichia coli* (Cronan *et al.*, 1982; Webb *et al.*, 2004). *E. coli* PanC encodes a pantothenate synthetase which catalyzes the last step of pantothenate biosynthesis (EC 6.3.2.1; Fig. 1), the ATP-dependent condensation of pantoate and β -alanine to yield pantothenate. The Mg²⁺-dependent reaction occurs in two steps (Fig. 1): pantoyl adenylate formation followed by nucleophilic attack on the activated carbonyl by β -alanine (Zheng & Blanchard, 2001).

Sar2676 is an acidic protein (MW 31 419 Da, pI 5.23) from MRSA strain 252 (Holden *et al.*, 2004) and has been annotated as pantothenate synthetase. This protein has been identified by two-

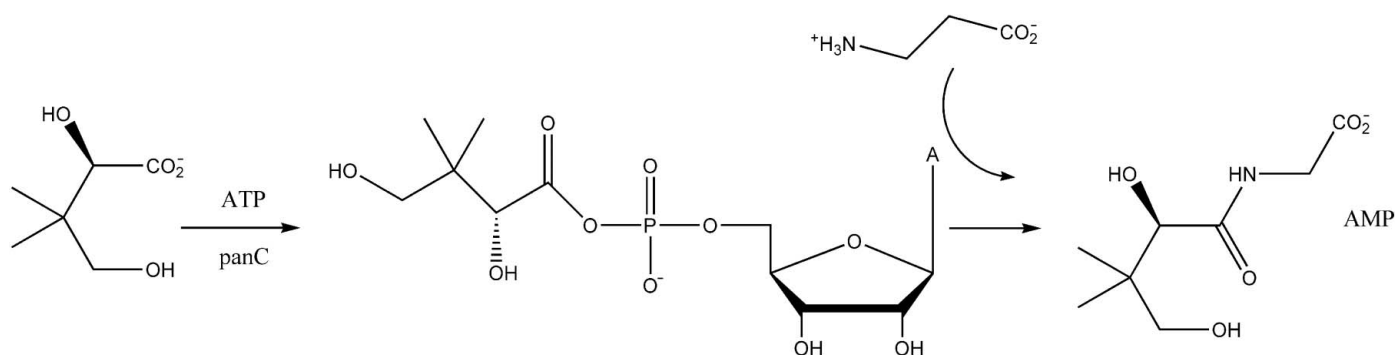


Figure 1

The reaction catalyzed by pantothenate synthetase.

dimensional gel proteomics as being upregulated in MRSA compared with a methicillin-sensitive strain of *S. aureus* (MSSA476). Sar2676 is approximately 40% identical to the known *E. coli* enzyme, the structure of which has been reported (PDB code 1iio; Von Delft *et al.*, 2001). Several complexes of the *Mycobacterium tuberculosis* enzyme have been reported (Wang & Eisenberg, 2003, 2006). Both the *E. coli* and *M. tuberculosis* enzymes are functional dimers. As part of our program on MRSA, we have cloned, expressed, purified and crystallized Sar2676 protein.

2. Materials and methods

2.1. Proteomics

Crude cell extracts were prepared as described by Oku *et al.* (2004) with modifications as described by Seetharamappa *et al.* (2007). A side-by-side image of two-dimensional gels with the spot identified as SAR2676 is shown in Fig. 2.

2.2. Cloning, expression and purification

The gene encoding Sar2676 was amplified from MRSA252 genomic DNA and cloned into pDEST14 as an N-terminal TEV protease-cleavable His-tag fusion using a modified Gateway recombination system (Liu & Naismith, unpublished work). The system adds a hexa-His tag and a TEV cleavage site at the N-terminus of the protein. The resulting plasmid was used to transform *E. coli* BL21 (DE3) (Novagen). 10 l of cells were grown in Luria broth with ampicillin (final concentration 100 µg ml⁻¹) at 310 K. When the cells reached an A_{600} of 0.6, 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added. After a further 6 h incubation period, cells were harvested by centrifugation at 2500g and 277 K for 30 min and then

resuspended in phosphate-buffered saline (PBS). The mixture was centrifuged as before and the cell pellets were stored at 193 K.

Sar2676 cell pellets were resuspended in lysis buffer (50 mM Na₃PO₄ pH 7.5, 500 mM NaCl, 1 mg ml⁻¹ lysozyme) with protease inhibitors and lysed on ice using a Constant Systems cell disrupter at 207 MPa. The crude lysate was clarified by centrifugation (15 000g, 30 min, 277 K) and filtered using a 0.22 µm filter. The target protein was bound to Ni Sepharose 6 Fast Flow media (GE Healthcare) and washed extensively with lysis buffer plus 20 mM imidazole. Sar2676 was eluted with lysis buffer plus 500 mM imidazole. Imidazole was removed by passage over a HiPrep 26/10 desalting column (GE Healthcare) with 50 mM Tris, 500 mM NaCl pH 7.5. The His tag was removed by incubation with TEV protease in a 1:10 ratio overnight for 15 h. The protease and uncleaved protein were removed by passage over Ni Sepharose 6 Fast Flow media and Sar2676 was further purified by S-75 gel filtration. The retention time was intermediate between that expected for a monomer and that for a dimer. The purified protein, which contains an additional N-terminal alanine residue, was characterized by SDS-PAGE and mass spectrometry before being concentrated to 23 mg ml⁻¹ in 10 mM Tris pH 7.5, 150 mM NaCl for crystallization.

2.3. Crystallization

Initial conditions for crystallization of Sar2676 were obtained using a sitting-drop vapour-diffusion screen of commercial sparse-matrix crystallization conditions (JMac, Nextal The Classics, Nextal pH Clear, EBS Wizard, Nextal JCSG and Nextal PEGs crystallization screens). Preliminary trials involved screening of protein solutions at concentrations of 11.5 mg ml⁻¹ with drop sizes of 0.2 or 0.3 µl (containing 0.1 or 0.2 µl protein solution and 0.1 µl precipitant

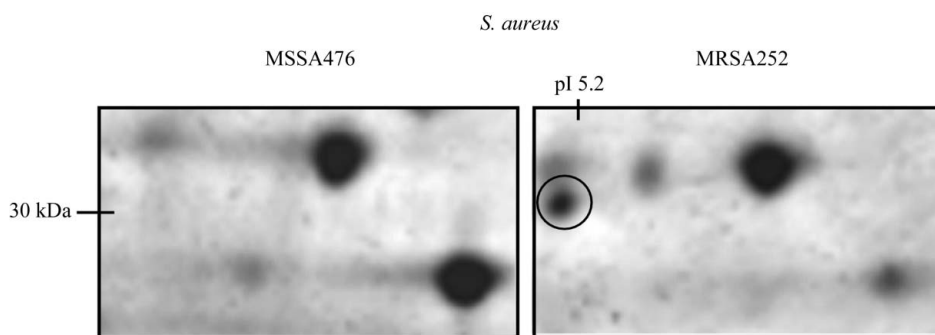


Figure 2

Crops from two-dimensional PAGE gels showing differences in protein expression between *S. aureus* MSSA476 and MRSA252. Crude membrane preparations were separated by IEF over the pH range 4–7 and proteins of interest were identified by LC-MS/MS. The circled protein was identified as SAR2676 (SwissProt: Q6GDK5). The experiment was performed in duplicate and a representative result is shown.

solution) and 23 mg ml⁻¹ with a drop size of 0.2 µl (containing 0.1 µl protein solution and 0.1 µl precipitant solution) at 293 K prepared using a nanodrop crystallization robot (Cartesian HoneyBee) as a part of the Hamilton–Thermo Rhombix system. Crystal trials were carried out in three Greiner square 96-well plates (Hampton Research, USA). Crystals were seen in 12 conditions (JMac, Nextal pH Clear and Nextal JCSG crystallization screens) using the Rhombix vision system. Based on the conditions under which the crystals grew, their size and their appearance, we decided to use two stochastic matrices of 24 conditions each using the method of Segelke (2001), one at high pH (8.5–9.5) and one at medium pH (6.0–7.0). The screens contained the following components: the stochastically combined precipitants PEG 8000, PEG 6000, PEG 4000 and PEG MME 2000, the buffers Bicine, Tris–HCl, CHES, HEPES, MOPS, sodium cacodylate and bis-Tris, the salts MgCl₂, MgSO₄, Li₂SO₄ and LiCl and the additives glycerol and ethylene glycol. The protein concentration was 11.5 mg ml⁻¹ and we employed the hanging-drop vapour-diffusion method (1 + 0.5 µl mixture of protein and crystallization solution equilibrated against 450 µl of the latter in a 24-well plate) at 293 K for the matrix experiments. Good-quality crystals, as judged by appearance, were observed in a number of conditions and were confirmed as protein by diffraction experiments. The optimum crystals from this fine screen, as judged by their size and regular shape, were obtained with precipitant consisting of 17.4% (w/v) PEG 8000, 0.1 M bis-Tris buffer pH 6.0 and 0.11 M MgCl₂ with a protein concentration of 11.5 mg ml⁻¹ in 10 mM Tris pH 7.5, 150 mM NaCl (Fig. 3). The crystals, which were obtained in 2–4 d, had a triclinic morphology (approximately 0.05 × 0.05 × 0.3 mm) and grew to full size in one week.

2.4. X-ray data collection

The crystals of Sar2676 were soaked for 5–10 s in a cryoprotectant solution which consisted of 0.1 M bis-Tris pH 6, 20% PEG 8000, 20% PEG 400. The soaked crystal was mounted on a cryo-loop (Hampton) and placed in a stream of nitrogen at 100 K for all further diffraction experiments. Diffraction data were collected using a MAR 225 CCD detector on beamline ID 23-2 at the European Synchrotron Research Facility (ESRF), Grenoble. The incident X-ray beam had a wavelength of 0.873 Å. 360 frames were recorded with a crystal-to-detector distance of 211 mm and non-overlapping 0.5° oscillations with 2 s exposure per image. A typical image is shown in Fig. 4. The



Figure 3
Crystals of Sar2676 of approximately 0.05 × 0.05 × 0.3 mm in size.

Table 1

Crystal data and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.873
Resolution (Å)	30.0–2.3 (2.4–2.3)
Space group	<i>P</i> 1
Temperature (K)	100
Detector	MAR 225 CCD
Unit-cell parameters (Å, °)	<i>a</i> = 45.3, <i>b</i> = 60.5, <i>c</i> = 117.6, <i>α</i> = 87.2, <i>β</i> = 81.2, <i>γ</i> = 68.4
<i>V</i> _M (Å ³ Da ⁻¹), 4 molecules	2.4
Solvent content in tetramer (%)	48
Unique reflections	48238
<i>I</i> / <i>σ</i> (<i>I</i>)	7.48 (2.04)
Average redundancy	1.7 (1.4)
Data completeness (%)	91.9 (76.2)
<i>R</i> _{merge} [†]	0.114 (0.33)

[†] $R_{\text{merge}} = \sum_i (\sum_j I_j - \langle I \rangle) / \sum_i (\sum_j I_j)$, where I_j is the intensity of the j th observation of reflection i , $\langle I \rangle$ is the mean of the intensities of all observations of reflection i , \sum_i is taken over all reflections and \sum_j is taken over all observations of each reflection.

data were integrated with the program *MOSFLM* (Leslie, 1992) and merged with *SCALA* (Evans, 1997) as implemented in *CCP4* (Collaborative Computational Project, Number 4, 1994). The initial indexing showed the crystals to be primitive triclinic, with unit-cell parameters *a* = 45.3, *b* = 60.5, *c* = 117.6 Å, *α* = 87.2, *β* = 81.2, *γ* = 68.4°. The program *POINTLESS*, implemented in *CCP4* (Collaborative Computational Project, Number 4, 1994), was employed to confirm that the space group was indeed *P*1. Data-collection statistics are given in Table 1.

3. Results and discussion

Calculation of the Matthews coefficient ($V_M = 2.4 \text{ \AA}^3 \text{ Da}^{-1}$) suggested the presence of four molecules of Sar2676 in the asymmetric unit and a solvent content of 48%. One, two, three, five and six molecules in the asymmetric unit gave Matthews coefficients of 9.4, 4.7, 3.2, 1.9 and 1.6 Å³ Da⁻¹ with solvent content of 87, 74, 61, 35 and

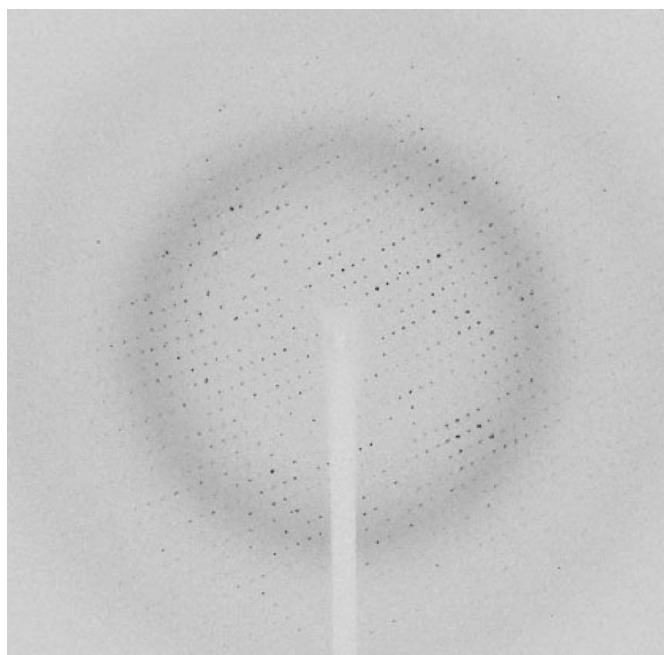


Figure 4
Diffraction pattern of Sar2676 collected at the ESRF.

22%, respectively. Self-rotation analysis does not identify a threefold, fourfold or fivefold rotation axis. The sequence match between the protein Sar2676 and pantothenate synthetase, its closest homologue in the Protein Data Bank (PDB code 1iho), is approximately 40% over the full protein length. Using a monomer of 1iho as a search model for molecular replacement gave a clear solution using *Phaser* (Storoni *et al.*, 2004; McCoy *et al.*, 2005) as implemented in *CCP4* (Collaborative Computational Project, Number 4, 1994). The final *Z* score was over 14 for four monomers in the asymmetric unit. The resulting solution packs sensibly in the unit cell and indicates that the protein adopts a dimeric arrangement, as observed for 1iho (Von Delft *et al.*, 2001). *Phaser* does not identify a fifth monomer. We are now rebuilding the model in order to correct the sequence and will report the final structure in due course.

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