Purification, crystallization and preliminary X-ray diffraction analysis of the *N*-acetyltransferase SAV0826 from *Staphylococcus aureus*

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Staphylococcus aureus is a prevalent microorganism that is capable of causing a wide range of infections and diseases. Several strains of this bacterial species have developed antibiotic resistance to methicillin and vancomycin, and higher death rates are still being reported each year owing to multidrug-resistant strains. Certain GCN5-related *N*-acetyltransferases (GNATs) exhibit a broad substrate range, including aminoglycosides, histones, other proteins and serotonin, and have been implicated in antibiotic drug resistance. Here, the expression, purification, crystallization and preliminary X-ray diffraction analysis of a GNAT from *S. aureus* (SaNAT) are reported. SaNAT was recombinantly expressed and crystallized by the hanging-drop vapour-diffusion method at 296 K, and the crystals diffracted to 1.7 Å resolution on the MX2 beamline at the Australian Synchrotron. The crystals belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 84.86$, $c = 49.06$ Å, $\alpha = \beta = \gamma = 90^\circ$. A single molecule is likely to be present in the asymmetric unit. A full structural and functional analysis is currently being undertaken to provide novel insights into the protein function, which in turn may provide a basis for drug design.

1. Introduction

Staphylococcus aureus is a Gram-positive, non-motile, non-spore-forming facultative anaerobic coccoid species. Infections may cause various diseases, ranging from minor skin infections (furuncles and boils) and eye infections (keratitis) through to life-threatening diseases including septic arthritis, pneumonia, bacteraemia, meningitis, osteomyelitis and endocarditis (Shittu & Lin, 2006; Trautmann *et al.*, 2002). The emergence of antibiotic resistance in *S. aureus* has led to increasing incidences of life-threatening infections, and certain strains of *S. aureus* have developed multidrug resistance towards essential antibiotics including methicillin, rifampicin and vancomycin (Cosgrove *et al.*, 2003; Stefani & Goglio, 2010). Approximately 19 000 deaths occur annually in the United States owing to methicillin-resistant *S. aureus* (MRSA) infections (Klevens *et al.*, 2007), and in 2011 ~80 000 invasive MRSA infections were reported in the United States (Dantes *et al.*, 2013).

N-Acetyltransferase activity has been shown to be one mechanism through which bacteria are able to develop antibiotic resistance. The enzymes that catalyze these reactions transfer acetyl groups from acetyl-CoA to primary amines or arylamines (Brooke *et al.*, 2003; Vetting *et al.*, 2005). Based on their cognate substrates and reaction mechanisms, they can be broadly categorized into either arylamine *N*-acetyltransferases (NAT1 and NAT2) or GCN5-related *N*-acetyltransferases (GNATs). Arylamine *N*-acetyltransferases have been shown to inactivate isoniazid, a front-line antitubercular agent (Sandy *et al.*, 2005), while enzymes of the GNAT family show diverse substrate specificity that includes aminoglycosides, histones and serotonin. Aminoglycoside *N*6'-acetyltransferases, members of the GNAT family, are responsible for the inactivation of aminoglycoside-containing antibiotics (Davies & Wright, 1997). GNAT members also exist in other emerging drug-resistant bacteria including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enterica* and *Streptococcus pneumoniae* and share identity ranging from 25 to 30%.

In addition to antibiotic modification, GNATs are responsible for a wide range of cellular functions through their recognition, interaction and acetylation of diverse substrates, and are one of the largest

enzyme families, with approximately 10 000 representatives from all kingdoms of life (Dyda *et al.*, 2000; Vetting *et al.*, 2005). The basic structure of the GNAT fold is highly conserved (Vetting *et al.*, 2005), sharing a common domain that is named after the yeast GCN5 and forms a structurally conserved fold consisting of six or seven β -strands (B) and four helices (H) in the topology B1–H1–H2–B2–B3–B4–H3–B5–H4–B6 followed by a C-terminal strand which may be from the same monomer or contributed by another monomer (*InterPro*; EMBL–EBI). In this communication, we report the 1.7 Å resolution crystal structure of an *N*-acetyltransferase from *S. aureus* (SaNAT) in the GNAT family. To date, this is one of the highest resolution structures to be solved in this family (1.7 Å), enabling a detailed structural analysis of key components including the active-site and substrate-binding pockets. Our structure may also assist in determining the structure–function relationships of GNATs in related pathogenic microbes including *Bacillus anthracis*, *S. pneumoniae* and *Clostridium botulinum* (27–38% sequence identity) owing to their universally conserved nature.

2. Methods

2.1. Cloning

The SaNAT gene *sav0826* was amplified using polymerase chain reaction (PCR) from genomic DNA (gDNA) of *S. aureus* subsp. *aureus* Mu50. The initial denaturation of gDNA was performed at 367 K for 15 min followed by 30 cycles of cyclic denaturation at 367 K for 1 min, annealing at 331 K for 1 min and extension at 345 K for 30 s; final extension was carried out at 345 K for 10 min. PCR-amplified product was purified using the Qiagen PCR purification kit, producing a single band on a 1% agarose gel. The forward and reverse primers used were TACTTCCAATCCAATGCCATGATTGAAATTAACATTAACGAATAATG and TTATCCAATTCCAAATGTTATTACTTATTAAATCAAAATCATAAAAAAGCC, respectively, in which the nucleotides in bold are complementary to the gene sequence and those in roman font are required for ligation-independent cloning (LIC). The purified amplicon was cloned into

SspI-digested and linearized pMCSG21 vector (Harvard Plasmid Repository) using T4 polymerase treatment (Eschenfeldt *et al.*, 2009). Clones were initially confirmed by colony PCR and the fidelity to the sequence was confirmed using DNA sequencing at the Australian Genome Research facility.

2.2. Expression and purification

SaNAT was recombinantly expressed as an N-terminally six-His-tagged fusion protein. A single colony of transformed *Escherichia coli* BL21 (DE3) pLysS cells was inoculated into 5 ml Luria–Bertani (LB) broth containing spectinomycin ($100 \mu\text{g ml}^{-1}$) overnight at room temperature (RT); 1 ml of this starter culture was added to 500 ml of auto-induction medium (Studier, 2005) in the presence of spectinomycin ($100 \mu\text{g ml}^{-1}$) and incubated at 298 K for 24 h in a shaking incubator. The cells were harvested by centrifugation at 5383g (Beckman Coulter Avanti J-E) and the cell pellet was resuspended in 30 ml His buffer A (50 mM phosphate buffer pH 8.0, 300 mM NaCl, 20 mM imidazole). Cell lysis was performed by three freeze–thaw cycles and the addition of lysozyme (1 ml of a 20 mg ml^{-1} stock). The cell debris was removed by centrifugation at 25 000g (Beckman Coulter Avanti J-E) and the supernatant containing the recombinant protein was subjected to affinity and size-exclusion chromatography. During affinity chromatography (HisTrap 5 ml HP, GE Healthcare), the His-tagged protein was applied to the column on an FPLC machine (AKTApurifier, GE Healthcare) and unbound proteins were removed through extensive washing (20 column volumes) in His buffer A. The protein was eluted from the column by applying a gradient of increasing imidazole concentration up to 500 mM in His buffer B (50 mM phosphate buffer pH 8.0, 300 mM NaCl, 500 mM imidazole) over ten column volumes. To cleave the six-His affinity tag from the protein, TEV protease was added at a final concentration of $15 \mu\text{g ml}^{-1}$ and incubated for 12 h at 276 K. This left three N-terminal residues encoded by the vector: Ser, Asp and Ala. The protein was further purified by size-exclusion chromatography (Superdex 200 column, GE Healthcare) in buffer consisting of 50 mM Tris pH 8.0, 125 mM NaCl, eluting at a volume consistent with a monomeric species. The protein was concentrated to 27 mg ml^{-1} using Millipore protein concentrators, the purity was assessed by SDS–PAGE (Fig. 1) and the protein was aliquoted and stored at 193 K in 50 μl aliquots for crystallization trials.

2.3. Crystallization

Crystallization trials were performed using the sparse-matrix hanging-drop vapour-diffusion method in 48-well plates, using a

Figure 1

Purification of recombinant SaNAT: lane 1, whole cell lysate after lysozyme treatment; lane 2, supernatant after centrifugation; lane 3, flowthrough of the fraction collected during affinity chromatography; lane 4, impurity peak fraction; lane 5, elution fraction after affinity chromatography; lane 6, TEV digestion of recombinant SaNAT; lane 7, elution after gel filtration; lane 8, pooled and concentrated SaNAT after gel filtration; lane 9, marker (labelled in kDa).

Figure 2

Diamond-shaped SaNAT protein crystals grown in 2.25 M sodium formate, 100 mM sodium acetate trihydrate pH 4.0 at 296 K.

Table 1

Data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Source	MX2, Australian Synchrotron
Radiation wavelength (Å)	0.9537
Data-collection temperature (K)	100
Space group	$P4_32_12$
Resolution range (Å)	30–1.7 (1.73–1.70)
Unit-cell parameters (Å, °)	$a = b = 84.86$, $c = 49.06$, $\alpha = \beta = \gamma = 90$
No. of measured reflections	270448 (14746)
No. of unique reflections	20192 (1057)
Completeness (%)	99.6 (100.0)
Multiplicity	13.4 (14.0)
Mosaicity (°)	0.55
Mean $I/\sigma(I)$	24.8 (9.6)
$R_{\text{p.i.m.}}$	0.029 (0.102)
Wilson B factor (Å ²)	20.90

range of crystallization screens from Hampton Research (Crystal Screen, Crystal Screen 2, PEG/Ion and PEG/Ion 2). Drops were set up manually by mixing the protein sample and reservoir solution together in a 1:1 ratio (total volume of 3 μl), suspended over reservoir solution (300 μl) and incubated at 296 K. To obtain X-ray diffraction-quality crystals, approximately 300 optimization drops were set up by changing the precipitant concentration and pH. Large diamond-shaped crystals that were readily reproduced (Fig. 2) and were suitable for diffraction were obtained in the condition 2.25 M sodium formate, 100 mM sodium acetate trihydrate pH 4.0.

2.4. Data collection and processing

Optimized crystals described above (§2.3) were flash-cooled in cryoprotectant comprised of the reservoir solution containing 30% glycerol. A single crystal was used to collect 360° of data on the MX2 crystallography beamline at the Australian Synchrotron (Fig. 3). Auto-indexing, merging and scaling of the diffraction data were performed using *iMosflm* (Battye *et al.*, 2011) and *AIMLESS* (Evans, 2006, 2011). Molecular replacement will be used to solve the structure of SaNAT.

3. Results and discussion

Recombinant SaNAT from *S. aureus* was overexpressed in *E. coli* BL21 (DE3) pLysS cells in auto-induction medium, producing approximately 50 mg protein per litre of bacterial culture. Purification of SaNAT to homogeneity was achieved through two chromatographic steps: Ni-affinity chromatography and size-exclusion chromatography. Proteolytic removal of the six-His tag by TEV protease treatment resulted in a 3 kDa reduction in the molecular weight (Fig. 1). Further purification using size-exclusion chromatography resulted in a protein of greater than 95% purity (Fig. 1) consistent in size with a monomer.

Screening of crystal-inducing conditions of SaNAT using the sparse-matrix hanging-drop vapour-diffusion method resulted in small diamond-shaped crystals in condition No. 34 (0.1 M sodium acetate trihydrate pH 4.6, 1 M sodium formate) of Crystal Screen. These crystals were optimized for size by changing the pH and the concentration of the precipitant, producing single, large diffraction-quality crystals in 2.25 M sodium formate, 100 mM sodium acetate trihydrate pH 4.0 at 296 K (Fig. 2). The crystals were stable and resistant to synchrotron-radiation damage throughout 360° of data collection (Fig. 3). SaNAT crystals diffracted to 1.7 Å resolution and the data frames were indexed, merged and scaled using *iMosflm* v.1.0.7 and *AIMLESS*, with $R_{\text{p.i.m.}}$ values of 2.9 and 10.2% over all

Figure 3
X-ray diffraction patterns at (a) 1° and (b) 360°.

resolution shells and in the outer resolution shell, respectively. The crystals belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 84.86$, $c = 49.06$ Å, $\alpha = \beta = \gamma = 90^\circ$. The data-collection statistics are summarized in Table 1. The Matthews coefficient V_M of 2.35 Å³ Da⁻¹ for one molecule in the asymmetric unit strongly suggests the presence of a single molecule (18 831 Da) in the asymmetric unit with 47.7% solvent content (Matthews, 1968). Structural and biochemical analysis are currently being undertaken.

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