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Cloning, expression, purification, crystallization and preliminary X-ray diffraction studies of *N*-acetylneuraminate lyase from methicillin-resistant *Staphylococcus aureus*

The enzyme *N*-acetylneuraminate lyase (EC 4.1.3.3) is involved in the metabolism of sialic acids. Specifically, the enzyme catalyzes the retro-aldol cleavage of *N*-acetylneuraminic acid to form *N*-acetyl-D-mannosamine and pyruvate. Sialic acids comprise a large family of nine-carbon amino sugars, all of which are derived from the parent compound *N*-acetylneuraminic acid. In recent years, *N*-acetylneuraminate lyase has received considerable attention from both mechanistic and structural viewpoints and has been recognized as a potential antimicrobial drug target. The *N*-acetylneuraminate lyase gene was cloned from methicillin-resistant *Staphylococcus aureus* genomic DNA, and recombinant protein was expressed and purified from *Escherichia coli* BL21 (DE3). The enzyme crystallized in a number of crystal forms, predominantly from PEG precipitants, with the best crystal diffracting to beyond 1.70 Å resolution in space group *P*2₁. Molecular replacement indicates the presence of eight monomers per asymmetric unit. Understanding the structural biology of *N*-acetylneuraminate lyase in pathogenic bacteria, such as methicillin-resistant *S. aureus*, will provide insights for the development of future antimicrobials.

1. Introduction

Staphylococcus aureus is a Gram-positive bacterial pathogen frequently found on the skin and in the nasopharynx of humans (Furuya & Lowy, 2006). Humans appear to be a natural reservoir for *S. aureus*; longitudinal studies have demonstrated that nasal colonization by *S. aureus* is persistent in approximately 20% (range 12–30%) of individuals, intermittent in around 30% (range 16–70%) of individuals and non-existent for about 50% (range 16–69%) of individuals (Eriksen *et al.*, 1995; Hu *et al.*, 1995; Kluytmans *et al.*, 1997; Nouwen *et al.*, 2004; Wertheim *et al.*, 2005). *S. aureus* has the potential to become extremely infectious, triggering a wide range of clinical diseases including skin and soft tissue infections, bloodstream infections, pneumonia, osteomyelitis and infective endocarditis (Furuya & Lowy, 2006). Higher rates of colonization and infection are often observed in patients with type I diabetes (Tuazon *et al.*, 1975), human immunodeficiency virus (Weinke *et al.*, 1992), intravenous drug users (Tuazon & Sheagren, 1974) and those receiving haemodialysis (Yu *et al.*, 1986).

Since Alexander Fleming's first observation in 1928 that penicillin had antibiotic properties against staphylococci (Hare, 1982), *S. aureus* has continuously evolved resistance mechanisms against novel antimicrobials (Furuya & Lowy, 2006). In 1959, the first semi-synthetic β -lactam antibiotic named methicillin was introduced. Despite initial successes, by 1961 the first methicillin-resistant *S. aureus* strains were emerging, which were resistant to virtually all β -lactam antibiotics and their derivatives (Jevons, 1961). As a result of this selective antibiotic pressure, there has been a dramatic increase in the number of methicillin-resistant *S. aureus* infections worldwide (Boyce *et al.*, 2005; Chambers, 1988; McDonald *et al.*, 1981; Panlilio *et al.*, 1992; Speller *et al.*, 1997). Previously associated with exposure in healthcare settings, this superbug now accounts for an increasing number of superadaptable community-acquired methicillin-resistant *S. aureus* infections (David & Daum, 2010). The ever-increasing prevalence of

Figure 1

Sialic acid metabolism. NanH, neuraminidase; NanT, transporter; NanA, lyase; NanK, kinase; NanE, epimerase; NagA, deacetylase; NagB, deaminase.

Figure 2

The reaction catalyzed by *N*-acetylneuraminase lyase.

methicillin-resistant *S. aureus* and the continual progression of antibiotic resistance highlight the need to investigate novel drug targets against this pathogen.

Sialic acids comprise a large family of nine-carbon amino sugars, all of which are derived from the parent compound *N*-acetylneuraminic acid (Almagro-Moreno & Boyd, 2009a; Vimr *et al.*, 2004). Interestingly, some pathogenic bacteria can catabolize sialic acid into carbon and nitrogen precursors by scavenging it from their surrounding environment (Almagro-Moreno & Boyd, 2009a; Vimr *et al.*, 2004). This sequestration and subsequent catabolism of sialic acid requires a well characterized cluster of genes confined to predominantly commensal and pathogenic species of bacteria, known as the 'Nan–Nag cluster' (Almagro-Moreno & Boyd, 2009a). The Nan–Nag cluster of genes encodes enzymes that scavenge sialic acid from the host, transport it into the bacterial cell and catabolize it into fructose-6-phosphate (Fig. 1). This pathway has been well documented in several bacterial pathogens such as *Escherichia coli*, *Haemophilus influenzae*, *S. aureus*, *Vibrio cholerae*, *Vibrio vulnificus* and *Yersinia pestis* (Almagro-Moreno & Boyd, 2009a,b; Chang *et al.*, 2004; Jeong *et al.*, 2009). All of these pathogens colonize heavily sialylated mucous-rich niches, such as the human respiratory tract and gut, suggesting that the ability to utilize sialic acid as a ubiquitous carbon and nitrogen source may be important for colonization and persistence (Almagro-Moreno & Boyd, 2009b). Importantly, a correlation between the Nan–Nag cluster of genes, pathogen colonization and pathogen persistence has been proven in mouse models for *E. coli* (Chang *et al.*, 2004), *V. cholerae* (Almagro-Moreno & Boyd, 2009b)

and *V. vulnificus* (Jeong *et al.*, 2009), making the pathway a viable and admirable target for antimicrobial drug design.

N-Acetylneuraminase lyase (also known as sialic acid aldolase) catalyzes the retro-aldol cleavage of *N*-acetylneuraminic acid (sialic acid, Neu5Ac) to form *N*-acetyl-D-mannosamine (ManNAc) and pyruvate (Fig. 2) via a Schiff base intermediate (Barbosa *et al.*, 2000; Izard *et al.*, 1994). The enzyme has recently been recognized as a potential antimicrobial drug target (Severi *et al.*, 2007; von Itzstein, 2007). The *N*-acetylneuraminase lyase subfamily of ($\beta\alpha$)₈-barrel enzymes all share a common structural framework, but catalyze different reactions on separate biochemical pathways (Lawrence *et al.*, 1997). Members of this subfamily identified thus far include the archetype *N*-acetylneuraminase lyase (Izard *et al.*, 1994), dihydrodipicolinate synthase (Kefala *et al.*, 2008; Burgess, Dobson, Bailey *et al.*, 2008; Dobson *et al.*, 2005; Evans, 2006; Mirwaldt *et al.*, 1995), D-5-keto-4-deoxyglucarate dehydratase (Jeffcoat *et al.*, 1969a,b), 2-keto-3-deoxygluconate aldolase (Buchanan *et al.*, 1999), *trans*-o-hydroxybenzylidenepyruvate hydratase-aldolase (Eaton, 1994) and *trans*-2'-carboxybenzalpyruvate hydratase-aldolase (Iwabuchi & Harayama, 1998).

On the basis of X-ray structural analysis, the *E. coli* *N*-acetylneuraminase lyase has been shown to be a homotetramer, with each subunit consisting of a ($\beta\alpha$)₈-barrel domain followed by an extension of three α -helices at the carboxy terminus (Izard *et al.*, 1994). Members of the *N*-acetylneuraminase lyase subfamily are all involved in the formation of a Schiff base between a strictly conserved lysine residue and the C2 carbon of an α -keto acid moiety on respective

substrates; further X-ray structural analysis of covalent complexes of *E. coli* *N*-acetylneuraminase with pyruvate and the substrate analogue 3-hydroxypyruvate provides evidence for this notion (Lawrence *et al.*, 1997). The three-dimensional structures of *N*-acetylneuraminase from *H. influenzae* in its native form and in complex with three substrate analogues (sialic acid alditol, 4-oxo-sialic acid and 4-deoxy-sialic acid) have also been determined by X-ray crystallography, revealing the mode of substrate binding within the *N*-acetylneuraminase active site (Barbosa *et al.*, 2000).

Understanding the structural biology of *N*-acetylneuraminase in methicillin-resistant *S. aureus* and other pathogenic species will provide insights necessary for the development of novel antimicrobial agent(s) that target the enzyme in question. Here we present the cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of *N*-acetylneuraminase from methicillin-resistant *S. aureus* subspecies *aureus* MRSA252.

2. Materials and methods

2.1. Cloning, expression and purification of *N*-acetylneuraminase lyase

The *nanA* structural gene encoding *N*-acetylneuraminase lyase and flanking nucleotide sequence were amplified by PCR using the primer pair SA_*nanA*-1F (5'-CGA TTG CGA TGT AAT ACA CC-3') and SA_*nanA*-1R (5'-CAT TGT AAA GGG GAT GTT GC-3') from genomic DNA derived from methicillin-resistant *S. aureus* subspecies *aureus* MRSA252. The amplified product was cloned into pCR-Blunt II-TOPO (Invitrogen) to produce a recombinant construct named pRN01. Following sequence verification, the primer pair SA_*nanA*_ORF-F (5'-CAT ATG AAC AAA GAT TTA AAA GG-3') and SA_*nanA*_ORF-R (5'-CAT ATG CTA TAA ATC GTA TTT TG-3') facilitated PCR amplification and cloning of the *nanA* open reading frame from pRN01 into the *Nde*I restriction site of the pET11a (Invitrogen, USA) expression vector to produce pRN02.

The recombinant expression vector, pRN02, was transformed into *E. coli* BL21 (DE3) cells which were cultured in Luria Broth media (with 50 µg ml⁻¹ ampicillin). Cultures were grown for approximately 5 h at 310 K and 200 rev min⁻¹, until an OD₆₀₀ of 0.6 was reached. Expression of *N*-acetylneuraminase lyase was induced by the addition

of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM, followed by further incubation at 310 K and 200 rev min⁻¹ overnight. Cells were harvested by centrifugation (8000 rev min⁻¹, 277 K, 5 min), resuspended in buffer consisting of 20 mM Tris-HCl pH 8.0 and lysed by sonication using a Vibra-cell VC 750 (SONICS). Cell debris was pelleted by centrifugation (10 000 rev min⁻¹, 277 K, 10 min).

Anion-exchange chromatography was performed using a Q Sepharose column (GE Healthcare), previously washed with three column volumes of 20 mM Tris-HCl pH 8.0. Cell lysate was loaded onto the Q Sepharose column and bound *N*-acetylneuraminase lyase was eluted using an increasing gradient to 20 mM Tris-HCl pH 8.0, 1 M NaCl. Hydrophobic interaction chromatography was implemented as the second purification step; 1 M ammonium sulfate was added to pooled fractions containing *N*-acetylneuraminase lyase from the anion-exchange step and then applied to a Phenyl Sepharose column (GE Healthcare) washed with three column volumes of 20 mM Tris-HCl pH 8.0, 1 M ammonium sulfate. Bound *N*-acetylneuraminase lyase was eluted using a decreasing gradient of ammonium sulfate concentration to 20 mM Tris-HCl pH 8.0. Size-exclusion chromatography was employed as the final purification step using a HiLoad 16/60 Superdex 200 column (GE Healthcare) with 20 mM Tris-HCl pH 8.0 buffer. All purification steps were carried out at 277 K. *N*-Acetylneuraminase lyase was concentrated using a 30 kDa molecular weight cutoff centricon (Millipore).

To determine the protein concentration after each purification step, the Bradford assay (Bradford, 1976) was used. A lactate dehydrogenase coupled assay was implemented to measure the catalytic activity of *N*-acetylneuraminase lyase for the reverse reaction, as described previously (Devenish & Gerrard, 2009).

2.2. Sequence analysis of bacterial *N*-acetylneuraminase lyase

A multiple protein sequence alignment was performed between *N*-acetylneuraminase lyase from *S. aureus* subspecies *aureus* MW2 (NP_645109) and four additional bacterial species [*Aggregatibacter aphrophilus* NJ8700 (YP_003007048), *Clostridium botulinum* D strain 1873 (ZP_04863430), *Gemella haemolysans* M341 (ZP_08259625), *Mannheimia haemolytica* serotype A2 strain Bovine (ZP_05988340.1)] as well as dihydrodipicolinate synthase from *S. aureus* subspecies *aureus* 71193 (YP_006195476) and *E. coli* strain K12 (NP_416973) (extracted from <http://www.ncbi.nlm.nih.gov>, protein accession numbers in parentheses). This alignment was performed using the program MUSCLE (Edgar, 2004), with manual editing.

2.3. Crystallization of *N*-acetylneuraminase lyase

Crystallization studies were initially conducted using a 10 mg ml⁻¹ preparation of methicillin-resistant *S. aureus* *N*-acetylneuraminase lyase in 20 mM Tris-HCl pH 8.0. Initial protein crystallization trials were performed at the CSIRO node of the Bio21 Collaborative Crystallization Centre (C3; <http://www.csiro.au/c3/>) using The PACT Suite and The JCSG+ Suite crystal screens (Newman *et al.*, 2005, 2008) at 281 and 293 K as previously described (Burgess, Dobson, Dogovski *et al.*, 2008; Dobson, Atkinson *et al.*, 2008; Voss *et al.*, 2009). Crystal screens were performed using the sitting-drop vapour-diffusion method with droplets consisting of 150 nl protein solution and 150 nl reservoir solution. Several of The JCSG+ Suite screen conditions produced crystals with a needle-like morphology at 293 K (Fig. 3).

Optimization of the initial hits was carried out using the hanging-drop vapour-diffusion method. The following conditions were varied

Figure 3
Crystals of methicillin-resistant *S. aureus* *N*-acetylneuraminase lyase. The scale bar indicates 0.5 mm.

Table 1

X-ray data-collection statistics for methicillin-resistant *S. aureus* N-acetylneuraminidase lyase.

Statistical values for the highest-resolution shells are given in parentheses. The Matthews coefficient and solvent content are based on eight monomers, with a molecular weight of 33 042.5 Da in the asymmetric unit.

Wavelength (Å)	0.95369
Number of images	360
Oscillations (°)	0.5
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 80.2, b = 108.6, c = 130.8 \beta = 89.9$
Resolution (Å)	32.3–1.70 (1.79–1.70)
Observed reflections	823916 (91713)
Unique reflections	242175 (33054)
Completeness (%)	98.5 (92.4)
$R_{\text{merge}}^{\dagger}$	0.101 (0.376)
$R_{\text{r.i.m.}}^{\ddagger}$	0.118 (0.457)
$R_{\text{p.i.m.}}^{\S}$	0.061 (0.254)
Mean $I/\sigma(I)$	8.0 (2.6)
Redundancy	3.4 (2.8)
Wilson B value (Å ²)	15.5
Molecules per asymmetric unit	8
V_M (Å ³ Da ^{−1})	2.16
Solvent content (%)	43

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. $\ddagger R_{\text{r.i.m.}} = \sum_{hkl} \{N(hkl) / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$. $\S R_{\text{p.i.m.}} = \sum_{hkl} \{1 / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th intensity measurement of reflection hkl , $\langle I(hkl) \rangle$ its average and N is the redundancy of a given reflection.

Table 2

Purification of recombinant methicillin-resistant *S. aureus* N-acetylneuraminidase lyase from a 1.5 l culture.

Purification step	Total protein (mg)	Specific activity (μmol min ^{−1} mg ^{−1})	Total activity (μmol min ^{−1})	Yield (%)
Crude	408	6.5	2646	100
Anion exchange	177	8.2	1443	55
Hydrophobic interaction	34.4	19	669	25
Size exclusion	32.8	11	374	14

for optimization: 1.7–2.2 μl of reservoir solution [The JCSG+ Suite condition H7: 25% (w/v) PEG 3350, 200 mM ammonium sulfate, 100 mM bis-tris pH 5.5] and 2.0 μl or 2.5 μl of protein solution (10 mg ml^{−1}, in 20 mM Tris–HCl pH 8.0) were equilibrated against 1 ml of reservoir solution in 24-well Linbro plates (Hampton Research) at 281 and 293 K. These conditions produced crystals in most wells.

2.4. Data collection and processing

For X-ray data collection, methicillin-resistant *S. aureus* N-acetylneuraminidase lyase crystals were briefly soaked in cryoprotectant solution containing reservoir solution made up to 20% (v/v) PEG 300 before being flash-cooled in liquid nitrogen. Intensity data were collected on the MX2 beamline at the Australian Synchrotron, Victoria, Australia (Fig. 4). Crystals that had been flash-cooled were mounted onto the beamline in a cold nitrogen stream at 110 K. The detector was positioned 169 mm from the crystal and data were collected in 0.5° steps for one 180° pass, with 90% attenuation and an exposure time of 2 s.

Indexing and integration of the data were performed using the program *iMOSFLM* (Battye *et al.*, 2011). The resulting intensity data were analysed using *POINTLESS* (Evans, 2006) from the *CCP4* program suite (Winn *et al.*, 2011) and *XTRIAGE* (Zwart *et al.*, 2005).

Scaling and data reduction were then performed using *SCALA* (Evans, 2006), also from *CCP4*. Molecular replacement was performed using the program *Phaser* (McCoy *et al.*, 2007) as implemented in *PHENIX* (Adams *et al.*, 2010). All relevant data-collection and processing parameters are given in Table 1. Images will be made available via the TARDIS server (Androulakis *et al.*, 2008) when the structure is published.

Figure 4

X-ray diffraction of methicillin-resistant *S. aureus* N-acetylneuraminidase lyase.

Figure 5

Purification of recombinant methicillin-resistant *S. aureus* N-acetylneuraminidase lyase. SDS-PAGE gel showing purification of methicillin-resistant *S. aureus* N-acetylneuraminidase lyase. Lane 1, molecular-weight markers (in kDa); lane 2, crude cell lysate; lane 3, pooled fractions from the anion-exchange chromatography step; lane 4, pooled fractions from the hydrophobic interaction chromatography step; lane 5, post-size-exclusion chromatography.

Figure 6
Sequence alignment of *N*-acetylneuraminase lyase from five bacterial species, as well as dihydrodipicolinate synthase from two bacterial species. Conserved residues are highlighted in pink. NAL denotes *N*-acetylneuraminase lyase and DHDPS denotes dihydrodipicolinate synthase.

3. Results and discussion

3.1. Expression and purification of *N*-acetylneuraminase lyase

Purification of *N*-acetylneuraminase lyase was carried out using anion-exchange chromatography, hydrophobic interaction chromatography and size-exclusion chromatography, yielding approximately 32.8 mg of pure protein with a specific activity of 11 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ from 1.5 l of bacterial cell culture (Table 2). The purity of the sample was at least 95% as estimated by SDS-PAGE (Fig. 5).

3.2. Sequence analysis of bacterial *N*-acetylneuraminase lyase

A multiple protein sequence alignment was carried out between *N*-acetylneuraminase lyase from *S. aureus* subspecies *aureus* MW2 and four additional bacterial species (*A. aphrophilus* NJ8700, *C. botulinum* D strain 1873, *G. hamemolysans* M341 and *M. haemolytica* serotype A2 strain Bovine) as well as dihydrodipicolinate synthase from *S. aureus* subspecies *aureus* 71193 and *E. coli* strain K12 (NP_416973). This was performed to investigate the

homology of *N*-acetylneuraminase lyase in *S. aureus* subspecies *aureus* MW2 with *N*-acetylneuraminase lyase in representative Gram-positive and Gram-negative bacterial species. The sequence alignment (Fig. 6) illustrates conservation of the active-site Lys165 (amino-acid numbering corresponds to that of *N*-acetylneuraminase lyase from *S. aureus* subspecies *aureus* MW2). It is proposed that this active-site Lys165 binds the α -keto acid moiety of the substrate in both *N*-acetylneuraminase lyase and dihydrodipicolinate synthase throughout all species studied to date (Barbosa *et al.*, 2000; Lawrence *et al.*, 1997). Other active-site residues also associated with the binding of the α -keto acid moiety of putative substrates, including the GXXGE motif, Ser47 and Ser(Thr)48 (that form the respective second and third residues of the GXXGE motif), and Tyr137 were conserved within *N*-acetylneuraminase lyase and dihydrodipicolinate synthase between species (Dobson *et al.*, 2004; Lawrence *et al.*, 1997). In addition to the conservation of these integral active-site residues, preservation of a salt bridge, Glu50 to Lys256, linking the C-terminal three-helical cluster and the $(\beta/\alpha)_8$ -barrel also seems to be highly conserved between species (Izard *et al.*, 1994). Residue I210, which in

dihydrodipicolinate synthases shows a conserved and strained non-planar peptide bond with the following serine residue (Dobson, Griffin *et al.*, 2008), is also conserved, although the serine (S209 in *E. coli* dihydrodipicolinate synthase) is replaced by a glycine in *N*-acetylneuraminase lyase enzymes, suggesting that this strain may be released. Based on the sequence alignment, five core residues of the β -barrel (Ala11, Ile77, Tyr137, Lys165 and Gly189) are invariant across both the *N*-acetylneuraminase lyase and dihydrodipicolinate synthase enzymes within all species (Izard *et al.*, 1994). This homology illustrates the importance of these residues and domains to the structure and function of the enzymes.

3.3. Crystallization, data collection and processing of *N*-acetylneuraminase lyase

As described in §2.3, several of The JCSG+ Suite screen conditions produced crystals with a needle-like morphology at 293 K (Fig. 3). In-house optimization of a successful condition (The JCSG+ Suite condition H7) selected from the high-throughput screening resulted in crystals that remained needle-like in morphology, although they were considerably larger in size. The best diffracting crystal was obtained from a 4.1 μ l droplet consisting of 2.1 μ l protein solution and 2.0 μ l reservoir solution. An X-ray diffraction data set to a resolution of 1.70 Å was collected from this crystal. This resolution is comparable to that of the wild-type unliganded structure of *N*-acetylneuraminase lyase from *H. influenzae* (1.60 Å; PDB entry 1f6k; Barbosa *et al.*, 2000) and the wild-type unliganded structure of *N*-acetylneuraminase lyase from *E. coli* (1.48 Å; PDB entry 3lbm; Chou *et al.*, 2011).

Analysis of the intensity data using *POINTLESS* indicated that the crystals belong to the space group $P2_1$. *XTRIAGE* suggested that the data were twinned, with twin law $h, -k, -l$. The l -statistic clearly suggests twinning with a value of 0.379 (untwinned = 0.5, perfect twin = 0.375). Initial phases were estimated by molecular replacement using the program *Phaser*. The monomer of *H. influenzae* *N*-acetylneuraminase lyase was used as the search model (PDB entry 1f6p; Barbosa *et al.*, 2000) and the Matthews coefficient (Matthews, 1968) was calculated to be 2.16 Å³ Da⁻¹ assuming eight monomers in the asymmetric unit, with a corresponding solvent content of 43%. The eight detected monomers appear to form two tetramers of similar monomer arrangement to that of other species of *N*-acetylneuraminase lyase. Molecular replacement with this search model using data scaled in $P2_1$ produced a final solution with a translation-function Z -score (TFZ) of 71.2. Refinement of the structure of methicillin-resistant *S. aureus* *N*-acetylneuraminase lyase is currently underway.

In summary, we present the cloning, expression, purification, crystallization and preliminary X-ray diffraction analysis of *N*-acetylneuraminase lyase from methicillin-resistant *S. aureus* subspecies *aureus* MRSA252. Understanding the structural biology of *N*-acetylneuraminase lyase in pathogenic species of bacteria, such as methicillin-resistant *S. aureus*, will provide us with the preliminary information necessary for future antimicrobial development.

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