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Cloning, overexpression, purification, crystallization and preliminary diffraction analysis of the receiver domain of MicA

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MicA is a response regulator from *Streptococcus pneumoniae* thought to be involved in redox-energy sensing under oxygen-limiting environments. The purified protein was crystallized using the sitting-drop vapour-diffusion technique. X-ray diffraction data were collected using synchrotron radiation to a resolution of 1.91 Å. The crystals belong to the monoclinic space group $C222_1$, with unit-cell parameters $a = 78.69$, $b = 92.57$, $c = 37.16$ Å, $\alpha = \beta = \gamma = 90.0^\circ$. The Matthews coefficient indicates that MicA crystallizes with one molecule in the asymmetric unit.

1. Introduction

Streptococcus pneumoniae is a bacterial pathogen responsible for diseases such as pneumonia, bacteraemia and meningitis and is the leading cause of illness and death in infants, the elderly and immunocompromized patients (Alonso DeValasco *et al.*, 1995). Many strains of *S. pneumoniae* are becoming resistant to a wide range of antibiotics, including vancomycin and erythromycin, and new targets for antibacterial agents are being sought.

Among such targets are the two-component signal-transduction systems, 13 of which exist in *S. pneumoniae*. At least one of them, MicAB, is essential for bacterial growth (Lange *et al.*, 1999; Throup *et al.*, 2000). Two-component systems, as the name suggests, are comprised of two distinct protein components, namely a histidine protein kinase (HPK), which is anchored to the cell membrane forming an extracellular sensor, and a cytoplasmic response regulator (RR). The latter has two distinct domains: a receiver domain, which accepts the phosphoryl group from the HPK on an aspartic acid, and a DNA-binding domain which controls gene expression (Stock *et al.*, 1995).

MicA, a 26 kDa protein, has been shown to be essential for bacterial growth as knockout mutations in these genes lead to non-viable bacterial strains, thus making it a prime target for new antibacterial agent design (Lange *et al.*, 1999). Studies on the CiaR/ComE system, a separate HPK/RR couplet in *S. pneumoniae*, have shown that these proteins are responsible for the mediation of competence (Echenique *et al.*, 2000). It has since been shown that the two-component signal-transduction system MicAB is involved in competence repression under oxygen-limiting environments (Echenique *et al.*, 2001). It was therefore proposed that the MicAB system, which contains a PAS domain (a protein–protein interaction region classically involved in redox reactions), was involved in the protection of the cell against oxidative stress, in particular by repression of competence. Under oxygen-limiting conditions, the MicB histidine kinase sensor phosphorylates MicA on a highly conserved aspartate residue in the presence of magnesium ions and MicA regulates an as yet unidentified gene.

In this paper, we report the cloning, purification, crystallization and preliminary crystallographic analysis of MicA from *S. pneumoniae*.

2. Experimental procedure and results

2.1. Cloning of MicA receiver domain

Using the TIGR4 strain genomic pneumococcal DNA as a template, the *MicA* gene was isolated using the polymerase chain reaction (PCR) with the following oligonucleotide primers: primer 1, TCCACCATGGGAAAAAAAATACTAATTGTA and primer 2, ATCATCCTCGAGTCATTGAGAACGACGCAG, with melting points of 310 and 316 K, respectively. The primers introduced *Nco*I and *Xho*I restriction sites to aid in cloning with the initiation sequence (ATG) within the *Nco*I site. Primers were designed in such a way as to avoid the encoding of a polyhistidine tag at the N- or C-terminus; thereby, only recombinant MicA was produced. In the 100 µl PCR reaction, 3 µl of genomic serotype 4 DNA was used as a template together with 1 µ*M* of each primer, 10 µl of Vent buffer (×10), 2 m*M* $MgSO_4$, 250 µ*M* of each deoxynucleotide triphosphate (dNTP) and 1 unit of Vent DNA polymerase (New England Biolabs). PCR reactions were performed at temperatures of 310, 312, 314 and 316 K. Using the amplified gene obtained from the 316 K cycle of PCR, the MicA DNA was ligated into pET33b vector

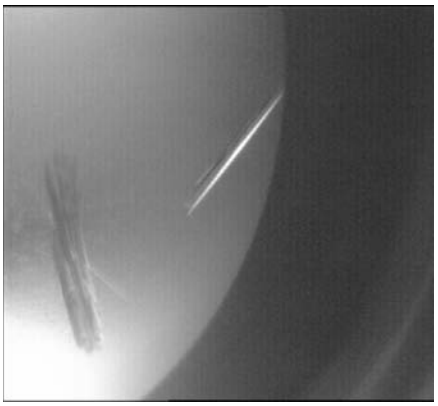

Figure 1
A crystal of MicA obtained using the conditions described in Table 1.

(Novagen), which utilizes the T7*lac* promoter to drive recombinant protein overexpression.

2.2. Protein expression and purification

Expression plasmids were transformed into the BL21 (DE3) *Escherichia coli* host strain (Stratagene) using a heat-shock technique. Several 1 l batches of LB-enriched growth media (Sigma) were inoculated with 10 ml of an overnight culture (Sambrook *et al.*, 1989). The cultures were shaken at 200 rev min^{-1} at 310 K until $OD_{600} \simeq 0.5$ was reached. IPTG (Melford) was then added to a final concentration of 1 m*M* in each culture to initiate overexpression of MicA. Cells were harvested at 5000*g* for 15 min at 277 K and resuspended in 10 ml buffer per litre of culture. The buffer consisted of 25 m*M* Tris pH 7.5 and 5 m*M* benzamidine (Sigma). Cells were lysed using sonication and DNaseI (Sigma) was added to a concentration of 20 µg ml^{-1}. Any insoluble debris was separated from the soluble protein by centrifugation at 10 000*g* for 30 min at 277 K.

MicA was initially purified using a Hi-Trap Q ion-exchange column (Amersham Pharmacia) previously equilibrated with 25 m*M* Tris pH 7.5 and was eluted using a NaCl gradient. Subsequent gel-filtration steps using Superdex-75 and Superdex-200 (Amersham Pharmacia) led to the purity of MicA reaching >95%. MicA is stable at 277 K for several months before any degradation is visible on silver-stained SDS–PAGE gels.

2.3. Crystallization and data collection

Crystallization trials were set up using both the sitting-drop and hanging-drop vapour-diffusion techniques at 293 K. 3.4 µl of purified protein at 7.2 mg ml^{-1} was mixed with 0.6 µl of the 1 ml reservoir solution. Initial crystallization trials for MicA were performed using Crystal Screens I and II (Hampton Research). MicA crystallized in condition No. 41 of Crystal Screen I [0.1 *M* Na HEPES pH 7.5, 10%(*v*/*v*) 2-propanol, 20%(*w*/*v*) PEG 4000] in 2 d. Screening around this condition yielded many crystals, none of which diffracted to beyond 4.5 Å.

Further trials were performed using the Emerald BioStructures Wizard I, Wizard II, Cryo I and Cryo II sparse-matrix screens. Several conditions gave crystals and screening around condition Nos. 5 and 47 in Cryo I and condition No. 18 in Cryo II yielded diffraction-quality crystals in 2 d. The crystals diffracted to beyond 2.5 Å in-house (Nonius FR591 generator operating at 100 kV and 50 mA, with 30 min exposures and a MacScience DIP2020 image plate).

Fresh crystals were cryocooled by quickly immersing them in a 100 K nitrogen-gas stream (Oxford Cryosystems) and were then stored in a liquid-nitrogen dewar for later use at a synchrotron source.

Crystals were tested on beamline 14.1 at the SRS, Daresbury with a fixed wavelength of 1.488 Å. A data set was collected at 100 K (Oxford Cryosystems) from a native crystal (Fig. 1) which diffracted to beyond 1.7 Å (Fig. 2). Data were collected at a distance of 100 mm from the crystal, with each frame 1° in width and using an exposure of 60 s. Owing to radiation damage suffered during the experiment, data were processed to a maximum resolution of 1.80 Å using *MOSFLM* (version 6.2.2; Leslie, 1992) and scaled using *SCALA* (Evans, 1993) and *TRUNCATE* (Collaborative Computational Project, Number 4, 1994). A summary of the crystallization conditions and X-ray data-collection statistics can be found in Table 1.

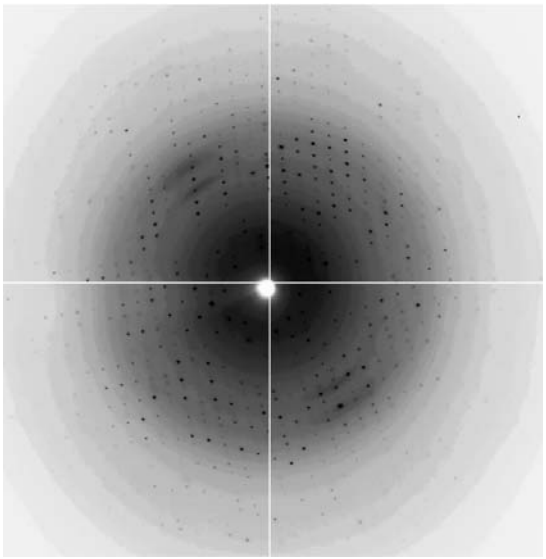

Figure 2
X-ray diffraction image of MicA receiver domain. Oscillation range, 1°; highest resolution, 1.67 Å.

Table 1
MicA crystallization conditions and X-ray data-collection characteristics.

Protein concentration (mg ml^{-1})	7.2
Crystallization solution	40%(*v*/*v*) PEG 400, citrate pH 5.5, 0.2 *M* $MgCl_2$ (final pH 4.6)
Unit-cell parameters (Å, °)	a = 78.69, b = 92.57, c = 37.16, $\alpha = \beta = \gamma = 90$
Unit-cell volume (Å^3)	270685.83
Crystal system	Orthorhombic
Space group	$C222_1$
Molecules per asymmetric unit	1
V_M (Å^3 Da^{-1})	2.53
Solvent content (%)	51.07
Upper diffraction limit (Å)	1.67

Table 2
Data-collection and processing statistics for MicA.

Values in parentheses refer to data in highest resolution shell.

Wavelength (Å)	1.488
Resolution (Å)	46.12–1.80 (1.90–1.80)
Total No. of reflections	126668
No. of unique reflections	12986
Completeness (%)	97.11 (85.3)
R_{sym} (%)	2.27 (10.9)
$I/\sigma(I)$	11.8 (4.0)

3. Results and discussion

Crystals of MicA measured 0.3 × 0.08 × 0.08 mm (Fig. 1) and diffracted to 1.67 Å resolution (Fig. 2). The unit-cell parameters are a = 78.69, b = 92.57, c = 37.16 Å, $\alpha = \beta = \gamma = 90°$ and the crystal belongs to the orthorhombic crystal system, space group $C222_1$, with a mosaicity of 0.49° at 100 K. With one monomer in the asymmetric unit, the determined Matthews coefficient is 2.53 Å^3 Da^{-1} (Matthews, 1968) and the solvent content is 51.07%. Both of these values are consistent with average crystal properties. A full data set with a completeness of 97.11% and an R_{sym} of 2.27% was collected (full data-processing statistics can be found in Table 2). Attempts are now

under way to solve the structure using molecular replacement with a starting model of PhoB chain *A* from *E. coli* (PDB code 1b00), which has 43% sequence homology to the receiver domain of MicA.

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