Crystallization and preliminary crystallographic analysis of an *Enterococcus faecalis* repressor protein, CylR2, involved in regulating cytolysin production through quorum-sensing

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CylR2 is one of two regulatory proteins associated with the quorum-sensing-dependent synthesis of cytolysin in the common pathogen *Enterococcus faecalis*. The protein was expressed with a C-terminal six-histidine tag and purified to homogeneity with a cobalt-affinity column followed by size-exclusion chromatography. Both native and SeMet proteins were produced and crystallized. Complete X-ray diffraction data sets were collected from a native crystal, which diffracted to 2.3 Å resolution, and a SeMet crystal, which diffracted to 2.1 Å. The crystals were tetragonal, belonging to space group $P4_1$ or $P4_3$, with unit-cell parameters $a = b = 66.2, c = 40.9$ Å, $\alpha = \beta = \gamma = 90°$. Based on the calculated Matthews coefficient of 2.6 $\text{Å}^3 \text{ Da}^{-1}$ as well as analysis of anomalous difference Patterson maps, the asymmetric unit most likely contains two molecules of CylR2.

1. Introduction

Enterococcus faecalis is among the most common opportunistic antibiotic-resistant pathogens found in hospital-borne infections (Callegan *et al.*, 2002; Coburn & Gilmore, 2003; Shepard & Gilmore, 2002). Unfortunately, the virulence of these enterococcal infections is greatly enhanced by the ability of as many as 60% of the clinical strains to secrete a novel cytolysin. This toxin is capable of lysing erythrocytes, destroying the neural tissue of higher organisms and killing a wide range of Gram-positive prokaryotic organisms. The active toxin consists of two post-translationally modified peptides, $CylL_L$ and $CylL_S$ (Booth *et al.*, 1996; Coburn & Gilmore, 2003). A cluster of genes has been identified as the cytolysin operon on a mobile conjugative plasmid, which can also be integrated into the chromosome of some *E. faecalis* strains within the pathogenicity islands (Gilmore *et al.*, 1994; Shankar *et al.*, 2002). These genes encode the peptide precursors of the toxin together with several other proteins involved in the modification and transportation of the mature toxin.

Cytolysin expression is controlled by two regulatory genes, *cylR1* and *cylR2*, located upstream from the cytolysin operon (Haas *et al.*, 2002). This regulation also involves a unique quorum-sensing mechanism. The shorter chain of the toxin peptide $CylL_S$ is identified as the autoinducer that mediates the production of the active cytolysin in a cell-density-dependent manner. At low cell density, cytolysin synthesis is repressed. This repression is overcome when the cell density reaches approximately 107 cells per millilitre. Genetic analysis indicates that both CylR1 and CylR2 are required for repression. The exact mechanism of this regulation is still unknown. CylR2 is a 66-residue protein. It is predicted to be a transcription factor with a helix–turn–helix DNA-binding motif that might be capable of interacting with the promoter region of the cytolysin operon for repression. Gapped BLAST and PSI-BLAST searches (Altschul *et al.*, 1997) against the known protein structures in the Protein Data Bank showed that CylR2 is remotely related to the SinR protein of *Bacillus subtilis*, with 19% sequence identity (Lewis *et al.*, 1998). As an initial step towards understanding the structural mechanism of these important regulatory proteins, we describe here the crystallization of CylR2 and its preliminary X-ray crystallographic analysis.

2. Methods and results

2.1. Cloning, expression and purification cylR2

The *cylR2* gene was amplified from *E. faecalis* genomic DNA (purchased from American Type Culture Collection) using a standard PCR protocol. The PCR primer sequences are 5′-TCAACAAGAGGA**GGTCTC**ACATGATAATCAATAAC and 5′-GTGAAATTTAAAAATCT**CTCGAG**TTCAGGTTGCCA, where the sequences in bold indicate the recognition sites for the restriction endonucleases *Bsa*I and *Xho*I, respectively. The resulting PCR product was digested with *Bsa*I and *Xho*I to generate a DNA insert with overhangs complementary to *Nco*I and *Xho*I sites on the vector and cloned into the expression vector pET28b (Novagen Inc.).

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DNA-sequencing data on the resulting expression plasmid (Seattle Biomedical Research Institute) indicated that the sequence of the clone was identical to the reported sequence of the *cylR2* gene (Francia *et al.*, 2001).

The plasmid containing *cylR2* was transformed into the expression host *Escherichia coli* Rosetta BL21(DE3) pLysS (Novagen) and the cells were grown at 310 K with vigorous shaking to an OD_{600} of ∼0.8 in 1 l Luria broth supplemented with 30 µg ml^{-1} kanamycin and chloramphenicol. Protein expression was induced by the addition of IPTG to a final concentration of 1.0 m*M*. At the same time, about 50 µl of 0.5 *M* zinc acetate was also added in case the protein bound to zinc, considering that a remote homolog, SinR, is known to bind zinc at its homodimer interface (Lewis *et al.*, 1998). The culture continued to be incubated with shaking for 4 h at 301 K. Cells were then harvested and stored at 193 K. For seleno-methionine (SeMet) labeling, *E. coli* B834 (DE3) pLys S cells transformed with the pET28b plasmid containing CylR2 were grown in 2 l of defined M9 medium supplemented with 50 µg ml^{-1} SeMet and other common amino acids. All other growth conditions for expressing the SeMet protein were similar to those for the native CylR2.

Thawed cells were resuspended in 35 ml lysis buffer (0.5 *M* NaCl, 20 m*M* Tris, 5 m*M* imidazole pH 7.8). Phenylmethylsulfonyl fluoride was added to the cell suspension to a final concentration of about 0.2 µ*M* immediately prior to cell lysis by three passes through a French press (SLM Instruments Inc.). The cell lysate was spun at 24 000*g* for 30 min. The supernatant was loaded onto a 10 ml cobalt-affinity column (BD Biosciences) and washed stepwise with 50 ml buffer (0.5 *M* NaCl, 20 m*M* Tris pH 7.8) with increasing concentrations of

Figure 1
Commassie-blue stained 4–20% SDS–tricine gel of CylR2 purified from the size-exclusion column. Lanes 1, 2 and 3, fractions of CylR2 from SEC; lane 4, molecular-weight markers.

imidazole (10, 50, 250 and 400 m*M*). The CylR2 protein was eluted from the cobalt column in several fractions of about 10 ml collected between 50 and 250 m*M* imidazole. Each fraction was analyzed on SDS–PAGE gel. Fractions with highly purified CylR2 were combined and dialyzed at 277 K overnight against a buffer containing 1 *M* NaCl, 20 m*M* Tris–HCl pH 7.8. The protein solution was concentrated with an Amicon Centriprep-3 followed by size-exclusion chromatography (SEC) on a Pharmacia Superdex75 HiLoad column equilibrated with the same buffer in order to maximize the monodispersity immediately prior to setting up crystallization trials. Following the cobalt-affinity column and size-exclusion chromatography, the C-terminal His-tagged CylR2 was purified to apparent homogeneity (Fig. 1). About 14 mg of purified proteins was obtained per litre of culture.

Pure CylR2 precipitated out at NaCl concentrations below 500 m*M*. NaCl greater than 800 m*M* was required for CylR2 to remain soluble. SEC indicated that at 8 mg ml^{-1} the majority of the protein appeared to form dimers in solution based on an elution volume of 74 ml at a flow rate of 1 ml min^{-1} on the Pharmacia Superdex 75 HiLoad column. Increased aggregation was observed at higher protein concentrations (>12 ml min^{-1}). Addition of either 10%(*w/v*) glycerol or 10 m*M* dithiothreitol (DTT) did not disrupt its aggregation, the latter being consistent with the absence of cysteine residues in the protein sequence. The SeMet protein behaved similarly to the native protein.

2.2. Crystallization of CylR2

Crystallization screening of CylR2 was carried out at room temperature (295 K) using the hanging-drop method. Drops were set up by mixing 2 µl CylR2 (10 mg ml^{-1}) with 2 µl of each reservoir buffer from the Hampton Research Crystal Screen kits. Showers of small clustered needle-shaped crystals occurred in several drops with PEGs of different molecular weights as the precipitants within the first week of incubation at 295 K. Conditions for crystallization were optimized around the initial successful buffers by varying the pH and the NaCl, polyethylene glycol (PEG) and protein concentrations. CylR2 crystallized as thin long clustered rod-shaped crystals with typical dimensions of 0.6–1.0 × 0.05–0.08 × 0.05–0.08 mm in a final crystallization buffer containing 30%(*w/v*) PEG 8000, 0.2 *M* sodium acetate, 0.1 *M* sodium cacodylate pH 6.5 and 12%(*w/v*) glycerol. However, these

long rod-shaped crystals diffracted poorly as they were easily damaged by the severe bending that occurred during crystal mounting and freezing. Hardier single crystals with more balanced dimensions were obtained by macroseeding (Fig. 2*a*). For macroseeding, crystals were briefly washed in the buffer and then transferred to a pre-equilibrated drop of the same buffer solution used to obtain the original crystals by mixing 2 µl of a diluted protein sample (about 8.5 mg ml^{-1}) with the crystallization buffer. Crystals were allowed to grow for approximately 2–3 weeks. Problems associated with crystal bending were avoided by cutting the long crystals into shorter fragments. The cut crystals were flash-cooled under liquid nitrogen. The SeMet CylR2 protein was crystallized under the same conditions as the native protein. The crystals were cryoprotected in the original crystallization solution containing 12% glycerol.

2.3. X-ray diffraction data collection and analysis

CylR2 crystals were initially screened and characterized using an in-house Bruker AXS Proteum 6000 X-ray diffractometer outfitted with a Cu rotating-anode X-ray source (FR-591) and a Smart6000 CCD
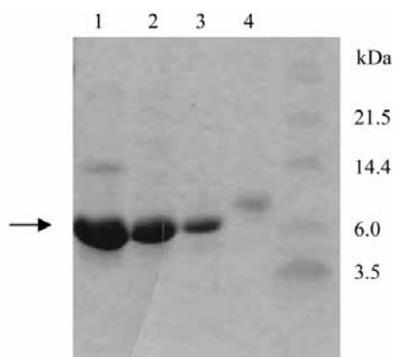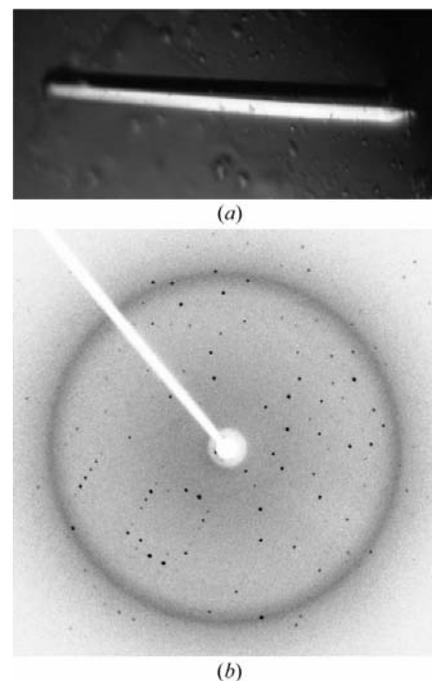
Figure 2
(*a*) Single crystal of native CylR2 grown in a buffer containing 30%(*w/v*) polyethylene glycol 8000, 0.2 *M* sodium acetate, 0.1 *M* sodium cacodylate pH 6.5 and 12%(*w/v*) glycerol after macroseeding. (*b*) The diffraction pattern of native CylR2 collected on the Bruker Proteum 6000 at a wavelength of 1.54 Å. The resolution at the edge of the image is 2.45 Å.

detector. A full data set was collected from a native CylR2 crystal at a crystal-to-detector distance of 60 mm under a steady stream of liquid nitrogen (100 K) with an exposure time of 30 s and an oscillation angle of 0.3° per image. Images were indexed, integrated and scaled using the Bruker *Proteum* software package (Bruker Advanced X-Ray Solutions, Madision, WI, USA). For SeMet-labeled crystals, data collection was performed at the Stanford Synchrotron Radiation Laboratory (beamline 8.3.1) and at Brookhaven National Laboratories (beamline X8-C). At the synchrotrons, each frame of data was collected using an oscillation range of 1° and a 1 s exposure. The data set was collected using inverse-φ geometry utilizing 20° wedges (that is, 20° of data were collected at φ and 20° of data were collected at $\varphi + 180$). An entire 360° sphere of data was collected from a single CylR2 crystal.

Optimized native CylR2 crystals typically diffracted to about 2.0–2.3 Å resolution either on the in-house diffractometer or at

synchrotron beamlines (Fig. 2*b*). A complete data set with a total of 1031 images was collected. Diffraction data statistics are summarized in Table 1. Indexing of the diffraction data showed that the crystals were tetragonal. From the complete data set, the space group was determined to be either $P4_1$ or $P4_3$, with unit-cell parameters $a = b = 66.2$, $c = 40.9$ Å, $\alpha = \beta = \gamma = 90°$. Analysis of the Matthews coefficient (Matthews, 1968) of 2.6 Å^3 Da^{-1} indicated that the crystallographic asymmetric unit probably contained two molecules of CylR2 and a solvent content of 51.5%. SeMet crystals had the same crystal system with similar unit-cell parameters.

CylR2 has only one methionine residue at the N-terminus and its presence in the purified protein sample was confirmed by the observation of a signature cross-peak for the methionine in a ^{1}H–^{13}C HSQC (heteronuclear single quantum coherence) NMR experiment (data not shown). Fluorescence measurements made at the synchrotron also confirmed the presence of selenium in the

Figure 4
Harker section ($z = 1/2$) of the CylR2 SAD data anomalous difference Patterson map. The map was calculated using data in the resolution range 15–4 Å. The Patterson map is contoured at 0.5σ levels with a 3σ cutoff. The Harker section shows two 4.5σ peaks representing the two Se atoms in the asymmetric unit.

SeMet crystal (Fig. 3). Preliminary analysis of anomalous difference Patterson maps from the single-wavelength anomalous diffraction (SAD) data collected at beamline 8.3.1 (ALS) show peaks for Se anomalous scatterers (Fig. 4), indicating that the SeMet was in the structured part of this 66-residue protein. This should allow us to determine the structure of CylR2 by MAD or SAD methods.

Table 1
Summary of crystallographic data of a native CylR2 crystal and an SeMet CylR2 crystal.

Values in parentheses refer to the highest resolution shell.

	Native†	SeMet SAD data‡
Source	Copper rotating anode, Bruker Proteum 6000	Beamline 8.3.1, ALS
Wavelength (Å)	1.54	0.979
Crystal system	Tetragonal	Tetragonal
Space group	$P4_1$ or $P4_3$	$P4_1$ or $P4_3$
Unit-cell parameters (Å)	$a = b = 66.2$, $c = 40.9$	$a = b = 66.53$, $c = 40.31$
Resolution range (Å)	50.0–2.3	50–2.05
Total No. observations	72850	412750
Total No. unique reflections	8079	21765
Completeness (%)	99.9 (99.4)	99.9 (100.0)
Redundancy	1.9 (1.8)	7.5 (7.5)
R_{sym} (%)	2.5 (22.1)	3.9 (30.3)
Average $I/\sigma(I)$	27.8 (4.1)	36.6 (5.3)

† Diffraction data statistics were obtained using the *XPREP* module of the *SHELX* software package (Sheldrick, 1998). ‡ Diffraction data were processed using the *DENZO* software package (Otwinowski & Minor, 1997).

Figure 3
Fluorescence scan for CylR2 crystals collected on beamline 8.3.1 at SSRL. SAD data were collected at a wavelength of 0.979546 Å, corresponding to an energy of 12 659 eV. This wavelength represented the *K* edge of the selenium incorporated into the selenomethionine as indicated by the fluorescence scan and hence is the optimal wavelength for the collection of SAD data.

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