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Expression, purification, crystallization and preliminary crystallographic studies of the *Enterococcus faecalis* cytolysin repressor CylR2

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The expression of an exotoxin called cytolysin contributes to the virulence of *Enterococcus faecalis*, one of the organisms responsible for antibiotic resistant infections acquired in hospitals. The DNA-binding protein CylR2 is a transcriptional repressor of cytolysin. At a specific cell density, cytolysin triggers signaling events, which result in the dissociation of CylR2 from its DNA-binding site. CylR2 was overexpressed in *Escherichia coli* and purified and crystals diffracting to 1.9 Å were obtained in two different crystal forms. One crystal form belongs to space group $P4_1$, with unit-cell parameters $a = 63.7$, $b = 63.7$, $c = 41.2$ Å, $\alpha = \beta = \gamma = 90^\circ$, and the other belongs to space group $P1$, with unit-cell parameters $a = 36.9$, $b = 45.0$, $c = 47.7$ Å, $\alpha = 67$, $\beta = 90$, $\gamma = 66^\circ$.

1. Introduction

Enterococcus faecalis is one of the major causes of antibiotic resistant infections acquired in hospitals (Murray, 1990; Jett *et al.*, 1994). It produces an exotoxin called cytolysin (Gilmore *et al.*, 1990) which attacks a variety of bacterial and mammalian cells. Cytolysin is expressed as a heterodimer consisting of a large and a small subunit, $CylL_L$ and $CylL_S$, respectively (Booth *et al.*, 1996). These subunits are post-translationally modified by the cytoplasmic protein CylM and then secreted by the ABC transporter CylB. In the extracellular space, the protease CylA activates cytolysin by cleaving the precursors and releasing the mature subunits $CylL_L''$ and $CylL_S''$. The protein CylI protects *E. faecalis* against the bactericidal activity of cytolysin (Haas & Gilmore, 1999). The genes for all these proteins, including those of cytolysin, were found on a collinear gene cluster (Gilmore *et al.*, 1994). Upstream, two more genes in opposite orientation, *cyl*R1 and *cyl*R2, were identified (Haas *et al.*, 2002). They encode two proteins able to almost completely repress expression from the cytolysin promoter pL (Haas *et al.*, 2002). CylR1, which is 94 amino acids in length, is predicted to be a membrane protein with three transmembrane helices. CylR2, which is 66 amino acids in length, is predicted to contain a helix–turn–helix DNA-binding motif. It was demonstrated that $CylL_S''$ alone was already sufficient to induce transcription from the pL promoter (Haas *et al.*, 2002). It was further found that derepression of the cytolysin operon was complete when the cell density reached a specific threshold (Haas *et al.*, 2002). This behavior is a typical feature of a quorum-sensing mechanism (Dunny & Winans, 1999). On the basis of these observations, a model was proposed in which CylR1 interacts with the DNA-binding protein CylR2, favoring transcriptional repression. As soon as cytolysin exceeds a specific concentration, it interacts with CylR1, changing the functional interaction between CylR1 and CylR2, which subsequently leads to the dissociation of CylR2 from the pL promoter.

We describe here the bacterial expression, purification, crystallization and preliminary X-ray analysis of native and iodide-soaked crystals of CylR2 as a first step towards structural information about this quorum-sensing system.

2. Cloning and expression

The coding sequence of CylR2 was amplified by PCR with *Pfu* Turbo DNA polymerase (Stratagene) and ligated between the *Nde*I and *Xho*I sites of the pET32a expression vector (Novagen). CylR2 expression was performed in *Escherichia coli* strain BL21(DE3). Cell cultures were grown at 310 K in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin. At an OD_{600} of 0.4–0.5, the cultures were switched to 301 K. When the cultures reached an OD_{600} of 0.6–0.7, protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 m*M*. The cultures were harvested after 6–7 h incubation at 301 K by centrifugation at 7500*g*.

3. Purification

The pellet from a 750 ml expression culture was suspended in 25 ml lysis buffer [50 m*M* HEPES pH 6.0, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* $MgCl_2$, 5 m*M* DTT, 0.5 m*M* PMSF with one tablet of Complete protease inhibitors

(Roche Diagnostics)]. The suspension was sonicated and the lysate was centrifuged at 48 000*g* for 45 min. The clear supernatant was filtrated through a 0.22 µm sterile filter and loaded onto a Hi-Trap Q-Sepharose (Amersham Biosciences) column (1 ml) equilibrated with lysis buffer. The flow-through and five column volumes of wash were pooled and loaded onto a Hi-Trap S-Sepharose (Amersham Biosciences) column (10 ml) equilibrated with lysis buffer. Elution was performed at a flow rate of 3.0 ml min^{-1} with a linear gradient of 0.1–1 *M* NaCl in the same buffer. Fractions containing CylR2 were pooled, flash-frozen in liquid nitrogen and dried by lyophilization. The pellet was taken up in a small volume of water and further purified by reversed-phase HPLC using an 8 × 250 mm semi-preparative C_{18}-HPLC column. The column was pre-equilibrated with H_2O/0.1% trifluoroacetic acid. Elution was performed by a linear gradient of 0–100% acetonitrile/0.1% trifluoroacetic acid. The purity of the eluted CylR2 was confirmed by electrospray mass spectrometry. The purified CylR2 was stored in lyophilized form at 253 K.

4. Crystallization

The first crystals grew spontaneously during room-temperature dialysis: CylR2 was dissolved at a concentration of 0.7 m*M* (5.4 mg ml^{-1}) in 50 m*M* HEPES pH 7.0 and 1.5 *M* NaCl and was dialyzed against the same buffer with an NaCl concentration of 0.3 m*M*. These first crystals took the form of irregular thin needles (Fig. 1*a*). Decreasing the temperature to 277 K only resulted in a delay of the appearance of crystals by ∼24 h; it did not improve their shape or size.

Microdialysis buttons (Hampton Research) were employed for a systematic search for better crystallization conditions; protein solution (50–60 µl) was placed in the chamber of each button, which was sealed with a semi-permeable membrane. Dialysis was performed against various buffers containing a range of NaCl concentrations in order to investigate optimal salting-in crystallization conditions. Addition of glycerol (10–30%) and stepwise transfer to solutions with decreasing salt concentrations slowed the rate of crystal growth and yielded thicker crystals that were suitable for X-ray analysis. Replacing NaCl with NaI did not yield better crystals. To obtain crystals suitable for X-ray analysis, the following crystallization protocol was employed: CylR2, dissolved at a concentration of 5–7 mg ml^{-1} in 1.5 *M* NaCl and 50 m*M* HEPES pH 7.0, was placed in microdialysis buttons, which were transferred every 24 h to new solutions, as shown in Table 1. When the microdialysis buttons had finally been transferred to the buffer with the lowest salt concentration, crystals started to appear after 6 h and grew in ∼1 d to dimensions of 0.9 × 0.05 × 0.05 mm (Fig. 1*b*).

Table 1
Crystallization conditions.

Condition No. 1: salting-in by stepwise transfer into the solutions below	Condition No. 2: vapor diffusion
1.5 *M* NaCl, 50 m*M* HEPES pH 7.0 1.5 *M* NaI, 50 m*M* HEPES pH 7.0 0.5 *M* NaI, 50 m*M* HEPES pH 7.0, 10% glycerol 0.2 *M* NaI, 50 m*M* HEPES pH 7.0, 10% glycerol	2.5 *M* AS, 10 m*M* $MgAc_2$, 50 m*M* MES pH 5.6

Crystals were also grown by the hanging-drop vapor-diffusion technique. The protein was dissolved at a concentration of 5.5 mg ml^{-1} in 400 m*M* NaCl, 50 m*M* HEPES pH 7.0 and 15 m*M* $MgCl_2$ and equilibrated against 2.5 *M* ammonium sulfate (AS), 10 m*M* magnesium acetate ($MgAc_2$) and 50 m*M* 2-(*N*-morpholino)-ethanesulfonic acid (MES) pH 5.6 (Table 1). Thin plate-like crystals with typical dimensions of ∼0.6 × 0.5 × 0.07 mm (Fig. 1*c*) appeared after 1 d.

5. Data collection and crystallographic analysis

All data sets were collected at 100 K with a MAR345 area detector using Cu $K\alpha$ radiation from a Siemens rotating-anode generator. A crystal grown under condition No. 1 was soaked for 30–60 s in mother-liquor solutions containing 30% glycerol and a total of 382° of data were collected. A crystal grown under condition No. 2 was soaked for a few seconds in cryoprotectant solution consisting of the mother liquor containing 13% glycerol. From this crystal, two native data sets were collected: one covering 282.5° and the other covering 170.5°. In order to collect the second data set, the crystal was rotated by ∼30° along the extended arc (Oxford Cryosystems). Data were processed by *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data-collection and processing statistics are reported in Table 2 (the two data sets collected from the crystal of crystallization condition No. 2 were scaled together).

Crystal form No. 1 is likely to contain a dimer in the asymmetric unit, giving a V_M value of 2.7 $Å^3$ Da^{-1} and 54% solvent content. The number of CylR2 molecules in the asymmetric unit of crystal form No. 2 is less obvious; two to four molecules per asymmetric unit give reasonable V_M values,

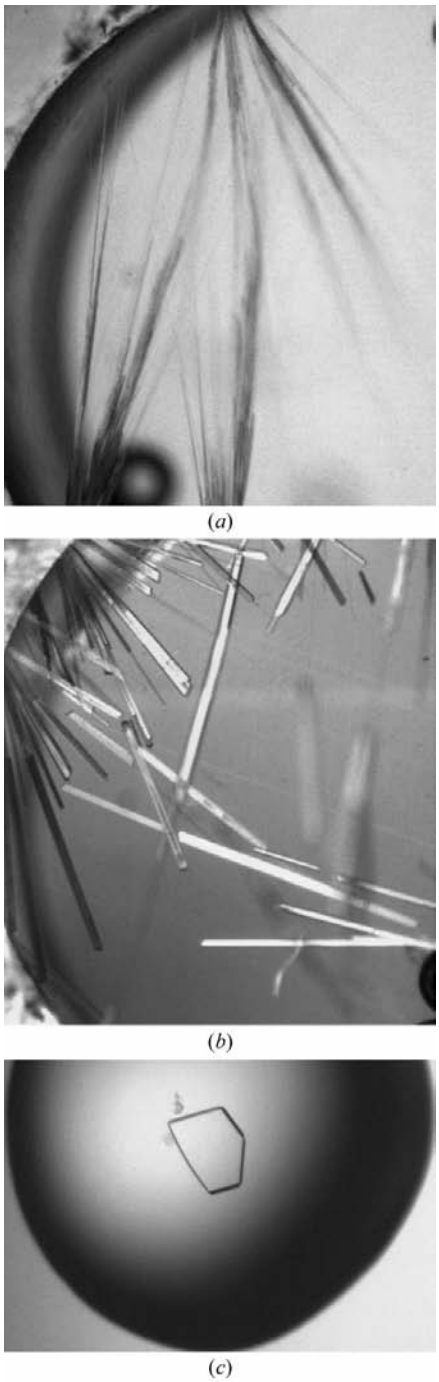

Figure 1
Crystals of CylR2. (*a*) Crystals obtained in a single step by dialyzing CylR2 from 1.5 *M* NaCl, 50 m*M* HEPES pH 7.0 to 0.3 *M* NaCl, 50 m*M* HEPES pH 7.0. (*b*) Crystals obtained by dialysing CylR2 stepwise. (*c*) Crystal grown by the hanging-drop vapor-diffusion technique with 2.5 *M* AS, 10 m*M* $MgAc_2$ and 50 m*M* MES pH 5.6.

Table 2
Data-collection statistics.

Values in parentheses correspond to the outer shell.

Crystal form	No. 1	No. 2
Space group	$P4_1$	$P1$
Resolution range (Å)	20–1.9 (2.00–1.90)	20–1.9 (1.97–1.90)
Unit-cell parameters		
a (Å)	63.68	36.93
b (Å)	63.68	44.98
c (Å)	41.19	47.70
α (°)	90.0	69.8
β (°)	90.0	89.6
γ (°)	90.0	66.1
Total No. of reflections	195718	67081
No. of unique reflections†	13086	19649
Completeness† (%)	99.2 (99.9)	96.1 (90.0)
Redundancy†	14.8 (13.2)	3.4 (2.8)
$R_{\rm merge}$†‡ (%)	10.4 (44.0)	5.8 (13.8)
Mean $I/\sigma(I)$†	19.0 (5.8)	19.0 (8.0)

† Friedel pairs merged. ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_i I_i(hkl)$.

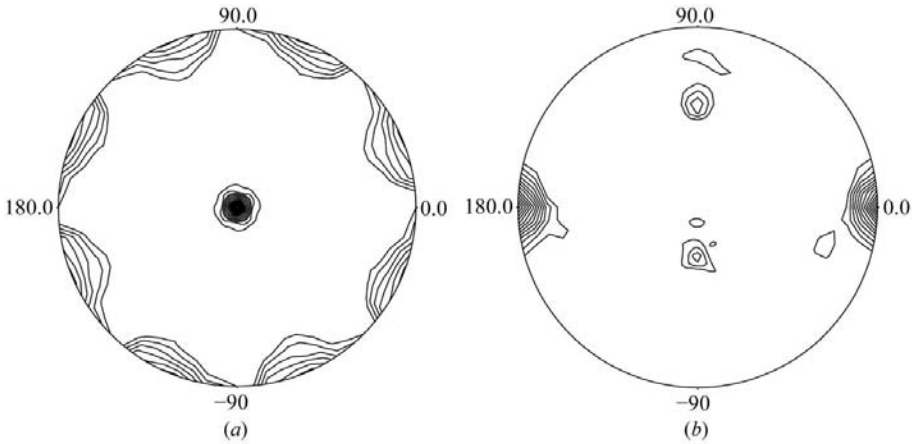

Figure 2
Self-rotation function, $\kappa = 180^\circ$ section. (*a*) Crystal form No. 1. (*b*) Crystal form No. 2. Radial coordinate, ω; angular coordinate, φ. The self-rotation function was calculated with reflections in the resolution range 14–2.5 Å and with maximum and minimum integration radii of 15 and 2 Å, respectively. Contour levels are plotted for all peaks greater than 25% of the origin peak in intervals of 5%.

ranging from 4.3 to 2.2 Å^3 Da^{-1}, and solvent contents of between 71.5 and 43%.

The Patterson self-rotation function was calculated for both crystal forms using *POLARRFN* from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994). In crystal form No. 1, in the $\kappa = 180^\circ$ section at $\omega = 90^\circ$, there are eight peaks at every 45° in φ at 51.3% of the origin peak (Fig. 2*a*). These peaks are generated by a non-crystallographic twofold axis perpendicular to the crystallographic fourfold axis, confirming the presence of a dimer. In crystal form No. 2, in the $\kappa = 180^\circ$ section there are three peaks at $(\omega, \varphi, \kappa) = (90, 180, 180^\circ)$, $(59, 90, 180^\circ)$ and $(31, 270, 180^\circ)$ at 88, 39 and 38%, respectively, of the origin peak (Fig. 2*b*). The strongest peak could relate two molecules in a dimer. The other two peaks are perpendicular to the strongest and may represent an additional non-crystallographic twofold axis, fulfilling the symmetry less well than the other axis. The latter axis may thus relate two dimers. Therefore, in crystal form No. 2 it is likely that there are four molecules in the asymmetric unit.

A *BLAST* search (Altschul *et al.*, 1997) for protein structures with a sequence related to CylR2 indicated two proteins, *Bacillus subtilis* SinR (Lewis *et al.*, 1998) and ribosome-recycling factor from *Thermus thermophilus* (ttRRF; Toyoda *et al.*, 2000), that have sequence identities of 31 and 32%, respectively. Molecular-replacement trials performed with both models and with fragments of them failed. Phasing by single anomalous dispersion (SAD) from non-covalently bound iodide ions (Dauter *et al.*, 2000; Dauter & Dauter, 2001) is currently under way. The anomalous signal of iodide ions is significant up to 2.5 Å resolution, as calculated by *XPREP* (Bruker AXS Inc., Madison, USA).

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