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Structural studies of the Hrp secretion system: expression, purification, crystallization and preliminary X-ray analysis of the C-terminal domain of the $HrcQ_B$ protein from *Pseudomonas syringae* pv. phaseolicola

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The C-terminal domain of the $HrcQ_B$ protein from the Hrp secretion system of the plant pathogenic bacterium *Pseudomonas syringae* pv. phaseolicola has been crystallized from MPD using the hanging-drop vapour-diffusion method. The crystals belong to space group $P2_1$, with unit-cell parameters $a = 51.6$, $b = 27.3$, $c = 97.2$ Å and $\beta = 99.8^\circ$. A complete native data set extending to 3.0 Å resolution was collected from a single cryoprotected crystal. The crystal solvent content and calculation of self-rotation functions showing non-crystallographic twofold symmetry axes are consistent with the presence of an oligomeric assembly in the asymmetric unit.

1. Introduction

The Hrp pathway is a type III protein-secretion system (Alfano & Collmer, 1997) used by several plant pathogenic bacteria to deliver death-triggering proteins to the interior of host cells. In non-host cells these pathogens use the Hrp system to deliver 'effector' proteins that activate the hypersensitive response (HR). The HR is a programmed cell-death process occurring at the site of invasion and is associated with the expression of plant defence. A similar type III transfer mechanism is used by the animal pathogens *Shigella*, *Salmonella* and *Yersinia* spp. to translocate virulence proteins into animal cells. The components of the Hrp system are encoded by the *hrp* genes. A subgroup of them known as *hrc* (*hrp* conserved) genes, shows sequence similarities with flagellar assembly genes and appear to be highly conserved among all bacteria that contain type III secretion systems (Galan, 1996; Rosqvist *et al.*, 1994; Hueck, 1998).

$HrcQ_B$, a low-molecular-weight (14 kDa) hydrophilic protein, is a component of the *P. syringae* Hrp secretion machinery and is encoded by a conserved *hrc* gene (*hrcQ*$_B$). In a model that has been proposed for the possible location and function of the Hrp proteins (based on the similarities of this secretion system with flaggelar assembly), $HrcQ_B$ is assumed to be a structural protein of the putative basal body of the Hrp apparatus and is located at the cytoplasm as its homologous FliN/Y (He, 1997).

The full-length $HrcQ_B$ protein is sensitive to proteolysis and has resisted all crystallization attempts. In this paper, we report the purification, crystallization and X-ray analysis of its proteolytically stable C-terminal domain that comprises 84 amino acids and has a molecular weight of 9 kDa (hereafter referred to as $HrcQ_B$fr). This fragment represents the conserved domain among $HrcQ_B$ homologues of other plant pathogenic bacteria and the flagellum FliY/N proteins and retains the ability of the full-length $HrcQ_B$ to interact with Hrp proteins (A. P. Tampakaki & N. J. Panopoulos, unpublished data). To our knowledge, this is the first protein from the Hrp secretion system that has been crystallized.

2. Experimental procedures

2.1. Expression and purification

The 270 bp DNA fragment that encodes the C-terminus of the $HrcQ_B$ protein (amino-acid sequence **MSYYHHHHHHDYDIPTTENLYFQ**GAMDPQDEPPALDSLALDLTLRCGELRLTLAELRRLDAGTILEVTGISPGHATLCHGEQVVAGEELVDVEGRLGLQITRLVTRS; the part in bold is removed after incubation with rTEV protease) was cloned into the pP_{RO}EX-HTa vector (Life Technologies, Inc.) and the product was expressed in *Escherichia coli* DH5a cells as a N-terminally His-tagged protein. For a typical preparation, 20 g of cell paste was resuspended in 180 ml buffer *A* (20 m*M* imidazole, 50 m*M* NaCl, 50 m*M* Na_2HPO_4/NaH_2PO_4 pH 8.0). Lysozyme was added to a final concentration of 1 mg ml^{-1} and the lysate was incubated on ice for 30 min. The disruption of the cells was completed by pulsed sonication for 4 min. After centrifugation (14 000*g* for 40 min) to remove the cell debris, the supernatant was loaded onto a 12 ml Ni-NTA column pre-equilibrated with 10 bed volumes of buffer *A*. The column was washed with 10 bed volumes of buffer *A*, 90 bed volumes of buffer 20 m*M*

imidazole, 300 m*M* NaCl, 50 m*M* Na_2HPO_4/NaH_2PO_4 pH 8.0; 40 bed volumes of buffer 40 m*M* imidazole, 300 m*M* NaCl, 50 m*M* Na_2HPO_4/NaH_2PO_4 pH 8.0 and 10 bed volumes of buffer 40 m*M* imidazole, 50 m*M* NaCl, 50 m*M* Na_2HPO_4/NaH_2PO_4 pH 8.0. The protein was eluted with 300 m*M* imidazole, 50 m*M* NaCl, 50 m*M* Na_2HPO_4/NaH_2PO_4 pH 8.0. The protein solution was dialysed against 50 m*M* Tris–HCl pH 8.0, 0.5 m*M* EDTA and was mixed with rTEV protease to remove the His tag. After 6 h of incubation at 303 K, the mixture was dialysed against 10 m*M* imidazole, 50 m*M* NaCl, 50 m*M* Na_2HPO_4/NaH_2PO_4 pH 8.0 and was loaded onto a 7 ml Ni-NTA column. The protein without the His tag was eluted during a wash with 10 m*M* imidazole. The protein solution was concentrated to 3 ml and applied to a 200 ml calibrated S-100 Sephacryl gel-filtration column pre-equilibrated with 20 m*M* Tris–HCl pH 7.5, 50 m*M* NaCl. The protein eluted in a volume that is consistent with the presence of a tetramer or pentamer (data not shown). The protein-containing fractions were pooled and concentrated to 8–10 mg ml^{-1}.

2.2. Crystallization

Crystallization conditions were initially screened with commercially available screens (Hampton Research, California, USA). Crystallization trials were performed by the conventional hanging-drop vapour-diffusion method (Ducruix & Giegé, 1992) using tissue-culture plates and siliconized glass cover slips (McPherson, 1982). Spherical arrangements of tiny needle crystals appeared in drops containing 30% MPD, 100 m*M* sodium cacodylate pH 6.5 and 200 m*M* magnesium acetate. This condition was refined by varying the pH and the type of buffer as well as the MPD and magnesium acetate concentrations. Clusters of thin plates emerging from a common origin were obtained with a reservoir solution composed of 13–15%(*v*/*v*) MPD, 70–90 m*M* magnesium acetate, 100 m*M* bis–tris pH 6.4–6.6. The crystallization drops were a mixture of 5 µl protein solution and 5 µl reservoir solution. The crystals appear within 7–10 d at 292 K.

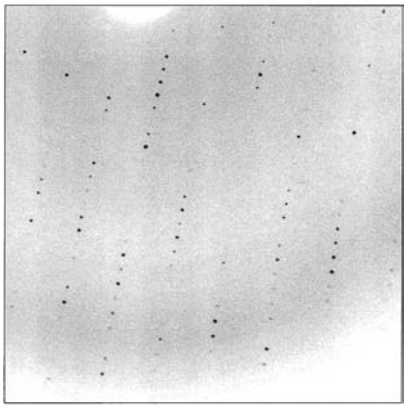

Figure 1
Detail from a typical diffraction pattern of $HrcQ_B$fr obtained with a laboratory source. The edge of the image corresponds to 3.0 Å resolution. (the image was prepared using the program *IPDISP* from the *CCP*4 suite of programs).

Table 1
Data-collection statistics for $HrcQ_B$fr protein.

	Overall (35.0–3.00 Å)	Highest resolution shell (3.11–3.00 Å)
R_{sym}	0.11	0.26
Completeness (%)	100	100
Multiplicity	4.6	3.7
No. of unique reflections	5675	553
No. of reflections	26299	2065
$I/\sigma(I)$	11.87	4.60

2.3. X-ray data collection

Owing to the sensitivity of the crystals to X-ray radiation, cryocooling was a necessary procedure during data collection in order to eliminate the crystal decay at room temperature. The crystals were transferred directly into a cryoprotectant solution containing 40% MPD, 80 m*M* magnesium acetate, 100 m*M* bis–tris pH 6.5, mounted in a nylon loop and flash-frozen in a nitrogen stream at 103 K. Data were collected on a MAR Research imaging plate with a diameter of 300 mm and a pixel size of 150 µm at the detector plane. The X-ray source was Cu $K\alpha$ radiation from a Rigaku RU-H3R rotating anode generator, focused and monochromated by a double-mirror system. Fig. 1 shows part of a typical diffraction image recorded from the $HrcQ_B$fr crystals. Intensities were integrated with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1997).

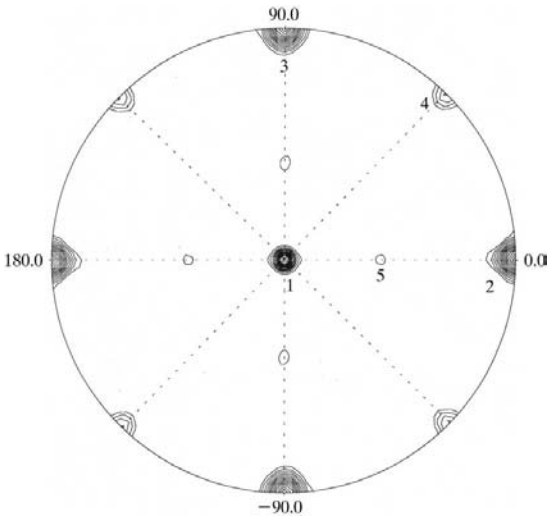

Figure 2
Section $\kappa = 180^\circ$ from the self-rotation function of $HrcQ_B$fr calculated using data in the resolution range 10.0–3.0 Å and a 17.0 Å radius of integration. The *b* axis is perpendicular to the plane of the paper and directed towards the viewer. The *c* axis is horizontal (corresponding to $\varphi = 0$, $\omega = 90^\circ$). The dashed lines represent four approximate mirror planes arising from the presence of non-crystallographic symmetry (see text for details).

3. Results and discussion

Although the crystals diffract to at least 2.2 Å resolution, the great majority of large crystals (about 0.2 × 0.6 × 0.02 mm) examined suffered from multiple splitting. To further improve the crystal quality we studied the influence of protein concentration, temperature, additives, detergents and seeding techniques. Some detergents (*n*-decyl-β-D-thiomaltoside and Fos-choline-12 from Hampton Research) and optimization of seeding had a beneficial effect on the growth of crystals as single thin plates. A crystal with dimensions 0.1 × 0.2 × 0.02 mm was used for data collection which only gave useful data to 3.0 Å resolution. The crystal had monoclinic symmetry with unit-cell parameters $a = 51.6$, $b = 27.3$, $c = 97.2$ Å, $\beta = 99.8^\circ$. Characteristic systematic absences and examination of the Harker sections of a native Patterson function indicated the space group $P2_1$. Statistics for the data collection are given in Table 1. Assuming three monomers per asymmetric unit, the Matthews coefficient, V_M (Matthews, 1968) is 2.4 $Å^3\ Da^{-1}$ (corresponding to a solvent content of ∼49%), while assuming four monomers per asymmetric unit, V_M is 1.83 $Å^3\ Da^{-1}$ and corresponds to ∼32% solvent. Both values are in the range found for proteins. Assuming two monomers per asymmetric unit, the V_M value (3.83 $Å^3\ Da^{-1}$) is close to the acceptable limits for proteins, although in terms of mechanical properties the crystals do not appear fragile enough to justify a solvent content of approximately 66%.

To test the presence of non-crystallographic symmetry, a self-rotation function was calculated using the program *POLARRFN* from the *CCP*4 suite of programs (Collaborative Computational Project, Number

4, 1994). Fig. 2 shows the section $\kappa = 180^\circ$ from a self-rotation function calculated using all data between 10 and 3 Å resolution and an integration radius of 17 Å. For the given space group ($P2_1$), there is just one anticipated (crystallographic) symmetry axis, which is marked as peak number 1 in Fig. 2. As is obvious, the rotation function indicates the presence of additional (non-crystallographic) twofold axes which are associated with a series of peaks at $\omega = 90^\circ$ and spaced every 45° on φ (peaks 2, 3, 4, ...), plus a few smaller peaks at lower ω values (for example, peak number 5). Additionally, the special location of those features (on planes parallel, perpendicular and 45° away from the crystallographic *bc* plane) creates an approximate 4*mm* symmetry for this section. Calculation of native Patterson functions using data from several different resolution ranges failed to reveal any outstanding non-origin peaks, thus indicating the absence of even-fold non-crystallographic symmetry axes parallel to the crystallographic twofold axis. Examination of the sections $\kappa = 90$ and 120° did not reveal the presence of threefold or fourfold axes. Taken together, the evidence from the self-rotation function and native Patterson functions are consistent with the biochemical data indicating that the protein is oligomeric in solution and suggest that the orientation of the non-crystallographic axes is special (parallel or perpendicular to the crystallographic axes and/or face or body diagonals).

A search for useful heavy-atom derivatives is in progress.

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