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Crystallization and preliminary crystal structure analysis of the ligand-binding domain of PqsR (MvfR), the *Pseudomonas* quinolone signal (PQS) responsive quorum-sensing transcription factor of *Pseudomonas aeruginosa*

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* employs three transcriptional regulators, LasR, RhlR and PqsR, to control the transcription of a large subset of its genes in a cell-density-dependent process known as quorum sensing. Here, the recombinant production, crystallization and structure solution of the ligand-binding domain of PqsR (MvfR), the LysR-type transcription factor that responds to the *Pseudomonas* quinolone signal (PQS), a quinolone-based quorum-sensing signal that is unique to *P. aeruginosa* and possibly a small number of other bacteria, is reported. PqsR regulates the expression of many virulence genes and may therefore be an interesting drug target. The ligand-binding domain (residues 91–319) was produced as a fusion with SUMO, and hexagonal-shaped crystals of purified PqsR_{91–319} were obtained using the vapour-diffusion method. Crystallization in the presence of a PQS precursor allowed data collection to 3.25 Å resolution on a synchrotron beamline, and initial phases have been obtained using single-wavelength anomalous diffraction data from seleno-L-methionine-labelled crystals, revealing the space group to be *P*6₅22, with unit-cell parameters *a* = *b* = 116–120, *c* = 115–117 Å.

1. Introduction

In order to effectively colonize a habitat, microorganisms need to coordinate their cellular activities not only to environmental cues but also to their own cell density. This is achieved through ‘quorum sensing’ (QS), a process that relies on self-synthesized diffusible small molecules called ‘autoinducers’ (AIs) that can cross the cell membrane in both directions and activate certain transcription factors (TFs) once a threshold concentration has been reached. Because AI-activated TFs also induce the transcription of AI synthase genes, a positive-feedback loop is generated that shifts metabolic energy from replication to the generation of molecules such as virulence factors that help the microorganism to further establish itself in the given environment (Antunes *et al.*, 2010). It is obvious that components of the QS machinery are potential drug targets in infectious diseases since their inhibition may reduce virulence and make the infectious agent more susceptible to the immune system of the host or to drugs (Njoroge & Sperandio, 2009).

Pseudomonas aeruginosa is a Gram-negative bacterium that causes a large number of opportunistic infections, especially in hospitals and in patients with cystic fibrosis, where it leads to chronic infection of the lungs and is the main cause of early mortality. *P. aeruginosa* infections are difficult to treat owing to the fact that the bacterium encodes a large number of efflux pumps for xenobiotics and forms robust biofilms in which it cannot easily be reached by pharmaceuticals (Høiby *et al.*, 2010). In addition, it produces a large arsenal of virulence factors that protect it against the immune system of the host and lead to tissue damage. Biofilm generation and the production of virulence factors are under QS control, which in *P. aeruginosa* involves three different AI/TF circuits that are arranged in a hierarchical manner and are interwoven into a network that also integrates other signals (Schuster & Greenberg, 2006). Of the three QS TFs, the LuxR-type family members LasR and RhlR are activated

by the *N*-acyl-homoserine lactones (AHLs) *N*-(3-oxododecanoyl)-L-homoserine lactone (C_{12} -AHL) and *N*-butyryl-L-homoserine lactone (C_4 -AHL), respectively. A third autoinducer, the 'Pseudomonas quinolone signal' 2-heptyl-3-hydroxy-4-quinolone (PQS) has recently been discovered (Pesci *et al.*, 1999). It binds and activates a TF originally named 'multiple virulence-factor regulator' MvfR (Cao *et al.*, 2001), which is now more often referred to as PqsR. PqsR contains 332 amino acids and belongs to the LysR family of TFs (also called LTTRs). These proteins possess a ligand-binding domain (approximately residues 90–320 in PqsR) that is connected *via* a long α -helix to a DNA-binding helix–turn–helix motif (residues 5–65 in PqsR). They associate into tetramers that are best described as dimers of dimers in which the initial dimerization is achieved by the interaction of two ligand-binding domains and the second dimerization occurs through antiparallel coiled-coil interactions along the α -helices that connect the ligand-binding and DNA-binding domains (Muraoka *et al.*, 2003; Zhou *et al.*, 2010). As a consequence, LTTRs contain four DNA-binding domains that recognize approximately 60 bp of DNA. In the case of PqsR, AI binding triggers transcription of the *pqsA–E* and *phnAB* operons, which are both required for PQS biosynthesis (Gallagher *et al.*, 2002). Localization and expression studies have suggested that PqsR is associated with the inner membrane and that it is deactivated by cleavage with translocation of an N-terminal fragment in the stationary phase (Cao *et al.*, 2001).

The PQS system seems to be unique to *P. aeruginosa* and a handful of related species (Diggle *et al.*, 2006). It acts in an intermediate fashion within the quorum-sensing network of *P. aeruginosa* by being activated by the *las* system and itself upregulating the *rhl*-regulated virulence factors through a mechanism the details of which are not yet fully understood (Fig. 1). Apparently, this mechanism involves the 'PQS-response protein' PqsE, a member of the metallo- β -lactamase family co-transcribed and therefore upregulated together with other enzymes required for PQS biosynthesis in a PqsR-dependent manner. The substrate of PqsE remains to be determined (Yu *et al.*, 2009), but it has been shown in three independent studies that both PqsE and the cognate *N*-acyl-homoserine lactone are required for RhIR-dependent virulence-factor production (Farrow *et al.*, 2008; Rampioni *et al.*, 2010; Hazan *et al.*, 2010). The transcription factor PqsR may therefore be an attractive drug target for new agents against *P. aeruginosa*, since it is almost unique to this pathogen and is required for the production of the indispensable factor PqsE.

A number of studies have investigated transcriptional regulation by PqsR and it has been demonstrated that it can also be activated by the PQS precursor 4-hydroxy-2-heptylquinolone (HHQ), albeit to a lesser extent than by PQS (Xiao *et al.*, 2006). Farnesol has been found to inhibit PqsR, which was attributed to the induction of a non-productive binding mode to the promoter sequence (Cugini *et al.*, 2007). Recently, it has been demonstrated that 2-aminoacetophenone, a volatile substance that gives *P. aeruginosa* cultures their typical smell, downregulates PqsR-dependent virulence processes (Kesarwani *et al.*, 2011) and the first synthetic inhibitors of PQS signalling have also been described (Lesic *et al.*, 2007; Lu *et al.*, 2012).

In order to improve opportunities for the design of inhibitors that block PQS signalling, structural insight into PqsR is highly sought. Very recently, Kefala and coworkers reported tetragonal crystals of a 242-residue C-terminal fragment that diffracted to 5 Å resolution (Kefala *et al.*, 2012). Here, we describe a hexagonal crystal form that is adopted by both the unlabelled and seleno-L-methionine-labelled ligand-binding domain of PqsR (residues 91–319) and that diffracts to 3.25 Å resolution.

2. Materials and methods

2.1. Cloning, expression and protein purification

Genomic DNA of *P. aeruginosa* strain PAO1 and sequence-specific DNA primers were used to amplify different stretches of the *pqsR* gene (*Pseudomonas* Genome Database entry PA1003; Winsor *et al.*, 2010). These PCR products were used for generation of expression plasmids using the services of the Dortmund Protein Facility (<http://www.mpi-dortmund.mpg.de/misc/dpf/index.htm>), which employs a recombination-based protocol (Oliner *et al.*, 1993; Li & Elledge, 2007). Correct integration into the plasmid backbones was confirmed by DNA sequencing. Protease-cleavable N-terminal His₆ tags with or without a downstream SUMO (small ubiquitin-related modifier) solubility enhancer fused to different fragments of PqsR (full length or fragments of the ligand-binding domain) were tested for expression and solubility in *Escherichia coli* under different expression conditions (IPTG induction at 293 or 310 K; autoinduction at 298 K). Only the SUMO fusions gave soluble proteins, but several, including the full-length construct, precipitated during the course of tag removal with SUMO protease. Therefore, subsequent work focused

Figure 1

Importance of PqsR in a simplified diagram of the *P. aeruginosa* quorum-sensing cascade. (1) The PQS system itself is activated by the LasR-*N*-(3-oxododecanoyl)-L-homoserine lactone (C_{12} -AHL) complex. (2) Binding of PQS to PqsR leads to autoinduction of the *pqs* operon. (3) PqsE enhances the activation of virulence genes (4) by RhIR-*N*-butyryl-L-homoserine lactone (C_4 -AHL) through an unknown mechanism.

on a fragment consisting of residues 91–319, which comprises the ligand-binding domain of PqsR. The forward DNA primer used for amplification of this fragment was 5'-GCGAACAGATCGGTGGTGGTCCGCGCAATCTCCGG-3' and the reverse primer was 5'-CGTGTCTAGAAAGCTTCAGCCTGAGCGGCGCTGC-3'. The resulting expression plasmid pOPIN-His-SUMO-PqsR_91-319 encodes a fusion protein consisting of an N-terminal His₆ tag linked to residues 2–98 of *Saccharomyces cerevisiae* ubiquitin-like protein SMT3 (SUMO; UniProt entry Q12306) followed by PqsR_91-319 (full sequence His₆-SUMO-PqsR_91-319: mgsshhhhhhgSDSEVNQ-EAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEA-FAKRQGMEDSLRFLYDGIQADQTPEDLDMEDNDIIEAH-REQIGG|GPRNLRVLLDTAIPPSFCDTVSSVLLDDFNMVSLIR-TSPADSLATIKQDNAEIDIAITIDEELKISRNFQCVLGYTKAF-VVAHPQHPLCNASLHSLASLANRYRQISLGRSGQHSNLLRPV-SDKVLFFVENFDDMLRLVEAGVGWGIAPHYFVEERLRNGTL-AVLSLEYEPGGIDTKVYCYNTALESERSFLRFLSARQRLR-ELGRQRFDDAPAWQPSIVETAQRRSG, where lower-case letters indicate the affinity tag, italic letters indicate SUMO and | indicates the SUMO protease cut site).

The plasmid was transformed into *E. coli* Rosetta2 pLysS cells and the cells were grown to an OD₆₀₀ of 0.7 at 310 K in Terrific Broth supplemented with 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol. The temperature was then reduced to 293 K and protein expression was induced with 0.5 mM IPTG for 16 h. The cells were then harvested by centrifugation, resuspended in buffer A (50 mM Na₂HPO₄, 300 mM NaCl pH 8.0) containing 1 mM PMSF and lysed by passage through a Microfluidizer (Microfluidics) three times. Insoluble matter was separated by centrifugation at 40 000g for 1 h and the resulting supernatant was filtered through a 0.2 µm filter before loading onto a HiTrap Chelating HP column (GE Healthcare) charged with 100 mM NiSO₄. The column was then washed with buffer A until the OD₂₈₀ was constant. Nonspecifically bound proteins were removed by washing with buffer A containing 5% buffer B (buffer A supplemented with 500 mM imidazole) before developing the column with a gradient to 100% buffer B. PqsR_91-319 eluted at 25–30% buffer B. Fractions containing pure His₆-SUMO-PqsR_91-319 were identified by SDS-PAGE and pooled. Protein concentrations were measured by UV absorption (calculated $\epsilon_{280} = 27.640 \text{ mol}^{-1} \text{ cm}^{-1}$) and 1 mg recombinant SUMO protease was added to 100 mg pooled protein to remove all of the residues of the His₆-SUMO tag from PqsR_91-319. Cleavage was allowed to proceed in a dialysis bag (cutoff 10 kDa) equilibrated against 50 mM HEPES, 150 mM NaCl pH 8.0 overnight at 277 K. The precipitate was sedimented by centrifugation and uncleaved protein was removed by passing the supernatant over a HiTrap Chelating HP column again. The flowthrough was collected, concentrated by ultrafiltration (Falcon UltraCentrifugal filter units, 10 kDa cutoff) and then applied to size-exclusion chromatography on a Superdex 75 26/60 column (GE Healthcare) equilibrated with 20 mM Tris-HCl, 150 mM NaCl, 5 mM β -mercaptoethanol pH 8.0. PqsR_91-319 eluted with approximately the same retention time as a 44 kDa molecular-weight standard (Supplementary Fig. S1¹), which is indicative of a homodimer (the calculated molecular weight of PqsR_91-319 is 52.1 kDa). The pure protein was concentrated to approximately 15 mg ml⁻¹ in the same buffer and flash-cooled in liquid nitrogen for storage at 193 K if not used immediately, since no differences in crystallization behaviour were noted between fresh and cooled protein samples.

¹ Supplementary material has been deposited in the IUCr electronic archive (Reference: EN5509).

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell. Both data sets were collected from a single crystal on beamline BL14.1 of the BESSY II synchrotron (Helmholtz Zentrum Berlin, Germany).

Data set	Se-SAD†	Native
Wavelength (Å)	0.97935	0.92526
Resolution range (Å)	48–3.40 (3.58–3.40)	46–3.25 (3.43–3.25)
Space group	<i>P</i> 6 ₂ 22	<i>P</i> 6 ₂ 22
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 120.9, <i>c</i> = 114.6	<i>a</i> = <i>b</i> = 116.9, <i>c</i> = 117.4
Mosaicity‡ (°)	0.157	0.161
Total No. of measured reflections	609714 (88480)	101880 (14797)
Unique reflections	7240 (1012)	7907 (1106)
Multiplicity	47.3 (47.4)	12.9 (13.4)
Mean <i>I</i> / σ (<i>I</i>)	25.0 (2.2)	19.2 (2.1)
Completeness (%)	100 (100)	99.9 (100)
<i>R</i> _{merge} § (%)	25.5 (383.5)	10.0 (153.8)
<i>R</i> _{p.i.m.} ¶ (%)	3.8 (56.3)	2.9 (43.0)
Optical resolution†† (Å)	2.56	2.44
Molecules per asymmetric unit	1	1
Matthews coefficient (Å ³ Da ⁻¹)	4.72	4.52
Solvent content (%)	73.9	72.8

† Friedel mates were treated as separate reflections. ‡ Mosaicity values are as reported by XDS (Kabsch, 2010). § $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation of the reflection with index *hkl*. ¶ $R_{\text{p.i.m.}} = \sum_{hkl} [1/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where *N* is the number of observations of the reflection with index *hkl* (Weiss, 2001). †† Optical resolution corresponds to the minimum distance of two peaks that are expected to be resolved in the electron-density map as calculated with SFCHECK (Vaguine *et al.*, 1999).

Seleno-L-methionine labelling was achieved by suppression of methionine biosynthesis (Doublié, 1997) in SeMet minimal medium (Guerrero *et al.*, 2001); the labelled protein was purified using the same strategy as that used for the native protein.

2.2. Crystallization, data collection and structure solution

Initial crystallization conditions of tag-removed PqsR_91-319 were identified by the sitting-drop vapour-diffusion method using JCSG Core Suites I–IV (Qiagen) and drops consisting of 150 nl protein solution and 150 nl mother liquor prepared using a nanodispensing robot (Phoenix, Art Robbins Instruments). The protein was screened at three different concentrations (7.5, 10 and 15 mg ml⁻¹) with or without pre-incubation with 4-hydroxy-2-heptylquinoline (HHQ), which was added to the protein solution to a final concentration of 5 mM from a 100 mM stock in DMSO. All crystallization experiments were performed at room temperature. Diamond-shaped crystals appeared using several precipitants, the compositions of which were subsequently optimized in 1 µl + 1 µl hanging drops equilibrated against 500 µl reservoir to generate larger crystals. The final precipitant in the case of native PqsR_91-319 in the presence of HHQ consisted of 0.2 M MgCl₂, 0.1 M Tris-HCl pH 8.4–8.6, 45% (v/v) ethylene glycol and the protein was employed at 15 mg ml⁻¹. The seleno-L-methionine-labelled protein gave crystals with a similar morphology, but the reservoir used for the data reported here consisted of 0.2 M LiCl, 0.1 M sodium citrate pH 5.6–5.8, 14–16% (v/v) ethanol. These crystals only appeared in the absence of HHQ.

Prior to data collection, the crystals were flash-cooled by plunging crystals mounted in nylon loops into liquid nitrogen. No cryoprotection was required for the native protein cocrystallized with HHQ, while the seleno-L-methionine derivative was briefly washed in mother liquor supplemented with 15% (v/v) glycerol. Diffraction data were collected using a Rayonics MX-225 CCD detector on beamline BL 14.1 of the BESSY II synchrotron (Helmholtz Zentrum Berlin, Germany; Mueller *et al.*, 2012). A native data set was collected as 220 non-overlapping 0.5° oscillation images at a wavelength of 0.92526 Å

(13.4 keV). For the seleno-L-methionine derivative, the X-ray energy was set to the approximate value of the Se *K* edge (12.659 keV) and single-wavelength anomalous diffraction (SAD) data were collected as 720 non-overlapping 1° frames. A fluorescence scan was not performed. Both data sets reported in Table 1 were indexed and integrated with *XDS* (Kabsch, 2010) and scaled with *SCALA* (Evans, 2006) from the *CCP4* package (Winn *et al.*, 2011) using default parameter settings.

Molecular replacement was attempted with *BALBES* (Long *et al.*, 2008), and SAD data from the seleno-L-methionine-labelled crystal were analysed using the *AutoSol* routine of *PHENIX* (Adams *et al.*,

2010). A sequence search with *BLAST* (Altschul *et al.*, 1990) and structure prediction with *PHYRE* (Kelley & Sternberg, 2009) identified PDB entry 3fd3 (Midwest Center for Structural Genomics, unpublished work) as the LTTR structure that was most similar to PqsR_91–319, and *MOLREP* (Vagin & Teplyakov, 2010) was used to place this model into the SAD electron-density map. For crystal-packing analysis, the smaller domain of PDB entry 3fd3 was adjusted by hand using *Coot* (Emsley *et al.*, 2010). Phasing of the native data applied a simple mask of this model constructed with *NCSMASK* followed by multi-crystal averaging with *DMMULTI* (Winn *et al.*, 2011). Further density improvement resulted from application of the ‘phase and build’ option in *PHENIX* (Adams *et al.*, 2010).

3. Results

PqsR is an important regulator of virulence in the human opportunistic pathogen *P. aeruginosa* and knowledge of its three-dimensional structure could significantly boost existing efforts towards inhibitor development (Cugini *et al.*, 2007; Lesic *et al.*, 2007; Kesarwani *et al.*, 2011; Lu *et al.*, 2012). In order to obtain sufficient amounts of protein for structural studies, we tested approximately 30 different PqsR fragment constructs for soluble expression in *E. coli*. Only fusion proteins bearing an N-terminal His₆-SUMO tag were soluble, including full-length PqsR, which however precipitated after tag removal with SUMO protease. It was therefore decided to focus on PqsR_91–319, which was predicted to contain the ligand-binding domain without a C-terminal structural element (residues 320–332) that is unique to PqsR. As expected, the protein eluted as a

Figure 2
Crystals of PqsR_91–319 grown in the presence of HHQ.

Figure 3
(a) The crystals shown in Fig. 2 typically showed split reflections. (b) Spurious diffraction misleads space-group determination. The pseudo-precession image was generated with *HKLVIEW* (Winn *et al.*, 2011).

Figure 4

(a) Experimental electron density after density modification, revealing a five-stranded β -sheet typical of LTTRs. (b) $P6_522$ crystal packing of PqsR_91–319 visualized by placing a related structure (PDB entry 3fd3; Midwest Center for Structural Genomics, unpublished work) into the electron density. This figure was generated with *PyMOL* (Schrödinger LLC).

homodimer in the final gel filtration; the overall yield was 15 mg PqsR_91–319 from 1 l cell culture. Seleno-L-methionine labelling was readily achieved by suppression of methionine biosynthesis, but required a SeMet minimal medium developed by Guerrero *et al.* (2001); labelling was not successful in M9 or LeMaster media.

Several initial crystallization conditions were identified quickly, but the same diamond-shaped crystal morphology was always observed despite relatively large variations in the precipitant compositions. Careful optimization led to the conditions indicated in §2.2. Interestingly, while addition of HHQ improved the appearance of crystals of the native protein, no crystals could be obtained when this ligand was included in crystallization of the seleno-L-methionine-labelled protein. PQS was also tested, but was not soluble under the conditions tested. With optimized precipitants, crystals appeared after 6 d and grew to dimensions of approximately $200 \times 150 \times 50 \mu\text{m}$ (Fig. 2).

Despite their perfect morphology, labelled and unlabelled crystals diffracted only weakly using a sealed-tube X-ray home source. This was not a consequence of the cryocooling procedure as capillary-mounted crystals at room temperature showed similar diffraction. Even at the synchrotron the maximum resolution that could be obtained was $3.25 \text{ \AA}$ in the case of native PqsR_91–319. Another problem was the split appearance of the reflections (Fig. 3a), which could be slightly improved by three rounds of 6 s freeze–thaw annealing of the worst (Supplementary Fig. S2) but not the best crystals, which are reported in Table 1. While the data could satisfactorily be indexed, integrated and merged in Laue class $6/mmm$, spurious diffraction on the *l* axis (Fig. 3b) hindered determination of the correct space group(s). However, the unit-cell parameters ($a = b = 116.9$, $c = 117.4 \text{ \AA}$ for the native crystal and $a = b = 120.1$, $c = 114.6 \text{ \AA}$ for the seleno-L-methionine derivative) suggested the presence of two protein chains in the asymmetric units at this stage.

Significant effort was required to extend analyses of these crystals beyond this primitive state. Molecular replacement was not successful, despite a large number of structures related to PqsR_91–319 being available in the PDB and exhaustive attempts testing monomeric and dimeric as well as sequence-edited models using the molecular-replacement engine *BALBES* (Long *et al.*, 2008). A breakthrough was achieved when initial phases to $3.7 \text{ \AA}$ resolution were obtained using SAD data from a seleno-L-methionine derivative

with *PHENIX* in the *AutoSol* mode (Adams *et al.*, 2010). The software located the four anomalous scatterers expected for the initially anticipated dimer. Initial phasing led to an overall FOM of 0.231, which increased to 0.778 after density modification and also revealed the correct space group to be $P6_522$, since only this enantiomorph resulted in connected electron density with protein-like features. In particular, the electron density showed the presence of a five-stranded β -sheet, which is a hallmark of the ligand-binding domain of LysR-type transcription factors (Fig. 4a). It was also possible to position the structure of a related protein (PDB entry 3fd3; Midwest Center for Structural Genomics, unpublished work) in this electron density, thereby strongly suggesting that the asymmetric unit contains only one protein chain, not the two that were initially expected from the unit-cell parameters. This also agrees with the finding that no noncrystallographic twofold axes could be detected by self-rotation search (Supplementary Fig. S3). Furthermore, the correspondingly high solvent content (73%; $V_M = 4.5 \text{ \AA}^3 \text{ Da}^{-1}$) is consistent with the relatively low resolution that was obtained (Kantardjieff & Rupp, 2003). Crystallographic symmetry thus generates the biological dimer of PqsR_91–319 and the packing produces solvent-filled channels along the *c* axis of the unit cell with an approximate diameter of $75 \text{ \AA}$ (Fig. 4b).

In order to transfer and extend the phases in the higher resolution native data set, we used cross-crystal averaging with *DMMULTI* (Winn *et al.*, 2011) and density modification through automatic model building in *PHENIX* (Adams *et al.*, 2010). This significantly improved the electron-density map such that the positions of the side chains became visible in many places. However, the automatically generated models are strongly fragmented and need to be completed by hand, which is time-consuming owing to the low quality of the electron density in some regions of the molecule. As a result, significant effort will also be invested in improving the current crystals and searching for new and better diffracting crystals.

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