The *Pseudomonas syringae* pv. *actinidiae* chemoreceptor protein F (PscF) periplasmic sensor domain: cloning, purification and X-ray crystallographic analysis

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Nitrate- and nitrite-sensing (NIT) domains are found associated with a wide variety of bacterial receptors, including chemoreceptors. However, the structure of a chemoreceptor-associated NIT domain has not yet been characterized. Recently, a chemoreceptor named PscF was identified from the plant pathogen *Pseudomonas syringae* pv. *actinidiae* that is predicted to contain a periplasmic NIT domain. The PscF sensor domain (PscF-SD; residues 42–332) was cloned into an appropriate expression vector, recombinantly produced in *Escherichia coli* BL21-Gold(DE3) cells and purified *via* immobilized metal-affinity and size-exclusion chromatography. Purified PscF-SD was screened for crystallization; the best crystal diffracted to a maximum resolution of 1.46 Å in space group $P2_12_12_1$. However, the data could not be phased using the only available NIT-domain structure (*Klebsiella oxytoca* NasR; PDB entry 4akk) as the search model. Therefore, a data set from a selenomethionine-labelled protein crystal was also collected. The selenomethionine-labelled protein crystal diffracted to a resolution of 2.46 Å in space group $P2_12_12_1$. These data will be used to attempt to solve the structure using the single-wavelength anomalous diffraction technique. The structure is expected to provide insights into the ligand specificity of NIT domains and the role of NIT domains in chemotaxis.

1. Introduction

Nitrate- and nitrite-sensing (NIT) domains are widespread (comprising an estimated ~3% of all known extracellular sensory domains in prokaryotes; Upadhyay *et al.*, 2016) and are found in receptors in a variety of different transduction pathways (Shu *et al.*, 2003). NIT domains were first proposed as specific nitrate and nitrite sensors through studies of the transcriptional regulator NasR from *Klebsiella oxytoca*. In NasR, an amino-terminal NIT domain is coupled to a carboxyl-terminal ANTAR (AmiR and NasR transcription antitermination regulator) domain (Boudes *et al.*, 2012). NasR selectively binds nitrate and nitrite *via* its NIT domain, and in the presence of these ligands it acts as a transcription anti-terminator (Goldman *et al.*, 1994; Lin & Stewart, 1996; Chai & Stewart, 1998). The crystal structure of NasR (PDB entry 4akk) revealed that the NIT domain is predominantly helical, forming a compact association of two four-helix bundles (Boudes *et al.*, 2012). Although the structure was obtained in the absence of nitrate or nitrite, ethylene glycol was found at the interface between the two bundles, flanked by two invariant arginine residues which were hypothesized to be a putative ligand-binding site.

Table 1

Macromolecule-production information.

The NdeI and XhoI restriction sites are underlined in the forward and reverse primers, respectively. The additional N-terminal residues of the tag, encoding a hexahistidine tag and a thrombin protease cleavage site, are underlined in the amino-acid sequence of the construct produced.

Source organism	<i>P. syringae</i> pv. <i>actinidiae</i> ICMP 18884
DNA source	Genomic DNA
Forward primer	<u>CAGCATATGG</u> ACTACCGCAGCAAGCTCGAT GTC
Reverse primer	CAGTCTCGAGTTAGAACTCCATGTTGGAGG TTCCATGG
Expression vector	pET-28a(+)
Expression host	<i>E. coli</i> BL21-Gold(DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHHSSGLVPRGSHMDYRSKLDVI SGEQSGVRQLLALDDLNVLSAQRDHAA RWKASDILREPTPAAKEAMANVDAGIPR ITKALEGVNAALIREAPADTMNRFKTL QAAVTGLDTASLRTVGWPDGYERFTTV LNLVQALREQIAMDSGLILDPWLETYML MQLSTQQVPDLIERIGRLSSVGQTSVAS GQFSLQSRQLQMRDLRGRIDDSKEQMNKA ASLLLSKLPEQLQPWADKYAATKQVLET ELKVIDDGVFGGSIKLPKEFEKSIDTL TGSTSDLRKQSLDALNGRLDYYHGTSNM EF

To date, NasR remains the only available NIT-domain structure. Recently, we identified a chemoreceptor named PscF from the plant pathogen *Pseudomonas syringae* pv. *actinidiae* that is predicted to contain a periplasmic NIT domain (McKellar *et al.*, 2015). Chemoreceptors play a key role in chemotaxis, the process by which microorganisms perceive chemical cues in their environment and move towards conditions that are favourable for survival (reviewed by Wadhams & Armitage, 2004). The periplasmic sensor domains of chemoreceptors are the input modules of a chemotaxis system, providing sensory specificity by binding particular ligands. Nitrate and nitrite are important sources of nitrogen for bacterial growth in a variety of habitats [for example the phyllosphere and rhizosphere (Parangan-Smith & Lindow, 2013; Dalsing & Allen, 2014), the ocean (Allen *et al.*, 2001) and soils (Myrold & Posavatz, 2007)], and taxis to nitrate and nitrite has been observed in several bacteria (Willey & Waterbury, 1989; Lee *et al.*, 2002; Dennis *et al.*, 2013). However, a dedicated chemoreceptor for nitrate/nitrite has not yet been characterized.

To gain insight into the ligand specificity of NIT domains and the role of NIT domains in chemotaxis, we aimed to determine the crystal structure of the PscF sensor domain (PscF-SD). Here, we report its cloning, expression, purification and preliminary X-ray diffraction analysis.

2. Materials and methods

2.1. Macromolecule production

The *pscF* gene encodes a predicted chemoreceptor with a NIT-type sensor domain (locus IYO_023035; accession No. AKT32348.1). The DNA fragment encoding the predicted periplasmic sensor-domain region of PscF (Asp42–Phe332) was amplified from genomic DNA using primers (Table 1) that

incorporated NdeI and XhoI restriction sites for cloning into pET-28a(+). The resulting vector was verified by DNA sequencing and was used to transform *Escherichia coli* BL21-Gold(DE3) cells for the expression of native PscF-SD and *E. coli* BL21(DE3) Δ *metA* cells for the expression of selenomethionine-labelled PscF-SD.

Native protein expression was carried out in Auto Induction Medium (Formedium) with 0.2% (w/v) glycerol and 30 μ g ml⁻¹ kanamycin sulfate. Cultures were grown at 37°C to an OD₆₀₀ of 0.6 before overnight incubation at 18°C. Selenomethionine-labelled protein was expressed in M9 medium (Formedium) supplemented with 255 μ M selenomethionine, trace elements (13.4 mM EDTA, 3.1 mM FeCl₃, 0.62 mM ZnCl₂, 76 μ M CuCl₂, 42 μ M CoCl₂, 162 μ M H₃BO₃, 8.1 μ M MnCl₂) and 30 μ g ml⁻¹ kanamycin sulfate. The cultures were grown at 37°C to an OD₆₀₀ of 0.6 before protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside and the cultures were incubated overnight at 18°C. The cells were harvested by centrifugation and resuspended in lysis buffer (50 mM potassium phosphate pH 7.0, 200 mM NaCl, 10% glycerol) with 5 U ml⁻¹ Benzonase nuclease, 4 μ l ml⁻¹ protease-inhibitor cocktail and 0.5 mg ml⁻¹ egg-white lysozyme. The resuspended cells were lysed by sonication on ice and the insoluble protein and cellular debris were pelleted by centrifugation. The His₆-tagged recombinant protein was purified from the soluble cell lysate using TALON metal-affinity resin. The protein was further purified using size-exclusion chromatography. Pooled elution fractions were injected onto a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated with crystallography buffer (50 mM HEPES, 200 mM NaCl pH 7.0). The protein fractions of interest were pooled and concentrated using Amicon Ultra-15 centrifugal concentrators (Millipore) with a molecular-weight cutoff of 10 kDa. Protein purity was assessed by SDS-PAGE (Fig. 1) and protein concentration was quantified by

Figure 1

Purified PscF-SD visualized with Coomassie Blue after separation on a 12% SDS-PAGE gel. Lane 1, molecular-size markers (Bio-Rad Precision Plus; labelled in kDa); lane 2, purified PscF-SD protein.

Table 2
Crystallization of PscF-SD.

	Native	Selenomethionine-labelled
Method	Sitting-drop vapour diffusion	Sitting-drop vapour diffusion
Plate type	96-well, SWISSCI 3 Lens Crystallization Plate (Hampton Research)	96-well, SWISSCI 3 Lens Crystallization Plate (Hampton Research)
Temperature (K)	291	291
Protein concentration (mg ml ⁻¹)	8.5	8.5
Buffer composition of protein solution	50 mM HEPES, 200 mM NaCl pH 7.0	50 mM HEPES, 200 mM NaCl pH 7.0
Composition of reservoir solution	0.1 M bis-tris propane pH 7.5, 0.2 M potassium thiocyanate, 20%(w/v) PEG 3350	0.1 M bis-tris propane pH 6.5, 0.2 M sodium formate, 20%(w/v) PEG 3350
Volume and ratio of drop	450 nl, 1:2	450 nl, 1:2
Volume of reservoir (μl)	40	40

measuring A_{280} ($\epsilon = 36\,440\text{ M}^{-1}\text{ cm}^{-1}$ for PscF-SD as calculated using *ProtParam*; Gasteiger *et al.*, 2005).

2.2. Crystallization

Crystallization trials were carried out with native and selenomethionine-labelled protein at a concentration of 8.5 mg ml⁻¹. Screening was performed against 288 crystallization conditions included in the PACT *premier* and JCSG-*plus* screens (Molecular Dimensions, UK) and Crystal Screen and Crystal Screen 2 (Hampton Research) using the sitting-drop vapour-diffusion method. Three protein:reservoir solution ratios (1:2, 1:1 and 2:1) were tested. The PACT *premier* screen produced crystals in several of the conditions. To confirm the identity of the crystallized protein, intact mass spectrometry was performed on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems) and LC-MS-based protein profiling was performed using nanospray LTQ-Orbitrap XL mass spectrometry coupled inline to an UltiMate 3000 nano-flow UHPLC (Thermo Fisher Scientific).

2.3. Data collection and processing

Native crystals from PACT *premier* condition G4 and crystals of selenomethionine-labelled protein from condition

F6 were flash-cooled in liquid nitrogen with the mother liquor providing cryoprotection. X-ray diffraction data sets were collected on the MX beamlines at the Australian Synchrotron (McPhillips *et al.*, 2002). A native crystal data set of 360° with 0.1° fine slicing was collected on MX2 at 70% beam attenuation to a resolution of 1.46 Å. For experimental phasing, a data set was collected from a selenomethionine-labelled protein crystal on MX1 at 12 000 eV ($\lambda = 0.968\text{ Å}$ and 50% beam attenuation) to a resolution of 2.46 Å. Data sets were indexed using *XDS* (Kabsch, 2010) and integrated and scaled with *AIMLESS* (Evans, 2011). Data-collection and processing statistics are given in Table 2.

3. Results and discussion

Purification of PscF-SD was carried out using immobilized metal-affinity chromatography and size-exclusion chromatography. SDS-PAGE showed that the purified protein migrated as a single band corresponding to the expected subunit mass (34.6 kDa; Fig. 1). Conditions G4 [0.1 M bis-tris propane pH 7.5, 0.2 M potassium thiocyanate, 20%(w/v) PEG 3350] and F6 [0.1 M bis-tris propane pH 6.5, 0.2 M sodium formate, 20%(w/v) PEG 3350] from the PACT *premier* screen produced

Figure 2
Typical PscF-SD crystals grown in (a) 0.1 M bis-tris propane pH 7.5, 0.2 M potassium thiocyanate, 20%(w/v) PEG 3350 and (b) 0.1 M bis-tris propane pH 6.5, 0.2 M sodium formate, 20%(w/v) PEG 3350.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

	Native	Selenomethionine-labelled
Diffraction source	MX2	MX1
Wavelength (Å)	0.946447	0.968600
Temperature (K)	100	100
Detector	EIGER	ADSC
Crystal-to-detector distance (mm)	200	250
Rotation range per image (°)	0.1	1
Total rotation range (°)	360	720
Exposure time per image (s)	0.02	1
Space group	$P2_12_12_1$	$P2_12_12_1$
a, b, c (Å)	48.20, 55.63, 111.81	49.09, 57.69, 114.50
α, β, γ (°)	90, 90, 90	90, 90, 90
Mosaicity (°)	0.12	0.15
Resolution range (Å)	48.2–1.46	45.12–2.46
Total No. of reflections	715787 (31385)	356268 (37810)
No. of unique reflections	53332 (2486)	12414 (1333)
Completeness (%)	99.8 (95.7)	99.8 (98.1)
Multiplicity	13.4 (12.6)	28.7 (28.4)
$\langle I/\sigma(I) \rangle$	22 (4.2)	22.1 (7.5)
$R_{\text{r.i.m.}}$	0.067 (0.649)	0.162 (0.591)

diffraction-quality crystals at 291 K after 13 and 21 d, respectively (Fig. 2). Mass spectrometry of the crystals confirmed that the intact molecular mass of the crystallized protein was 34.6 kDa, which corresponds to the molecular weight calculated from the primary sequence. The crystals were also analyzed by peptide fingerprinting, which confirmed the major component of the crystals to be PscF-SD, with 74.6% sequence coverage.

Isotropic diffraction data were collected to beyond 1.46 Å resolution for the native crystal, and the space group was predicted to be $P2_12_12_1$ (Table 3, Fig. 3). The protein has a molecular weight of 34.6 kDa and a Matthews coefficient of $2.33 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to 47.13% solvent content and a prediction of one molecule in the asymmetric unit. The data set was unable to be adequately phased by molecular replacement using its closest homologue the *K. oxytoca* NasR protein (PDB entry 4akk; Boudes *et al.*, 2012) as a model, with an LLG of 43 and a TFZ of 4.9. The solution was refined using *REFMAC5* from the *CCP4* suite (Murshudov *et al.*, 2011; Winn *et al.*, 2011), giving an *R* factor and *R*_{free} of 0.5661 and 0.5806, respectively. The secondary structure of the post-refinement model was superimposable with the 4akk structure; however, the side-chain electron density was uninterpretable. This inability to phase the data may be owing to the low sequence identity (~14%) between PscF-SD and NasR. In order to solve the above problem, we collected a data set from a selenomethionine-labelled crystal for experimental phasing (Table 3, Fig. 3). The data have an anomalous correlation of 0.39 at 3.3 Å resolution and these data will be used to attempt to solve the structure using the single-wavelength anomalous diffraction technique.

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Figure 3

Diffraction images from (a) the native protein crystal showing diffraction to a resolution of 1.46 Å and (b) the selenomethionine-labelled protein crystal showing diffraction to a resolution of 2.46 Å.

of the Australian Cancer Research Foundation (ACRF) detector installed on MX2.

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References

- Allen, A. E., Booth, M. G., Frischer, M. E., Verity, P. G., Zehr, J. P. & Zani, S. (2001). *Appl. Environ. Microbiol.* **67**, 5343–5348.
- Boudes, M., Lazar, N., Graille, M., Durand, D., Gaidenko, T. A., Stewart, V. & van Tilbeurgh, H. (2012). *Mol. Microbiol.* **85**, 431–444.
- Chai, W. & Stewart, V. (1998). *J. Mol. Biol.* **283**, 339–351.
- Dalsing, B. L. & Allen, C. (2014). *J. Bacteriol.* **196**, 949–960.
- Dennis, P. G., Seymour, J., Kumbun, K. & Tyson, G. W. (2013). *ISME J.* **7**, 1661–1664.
- Evans, P. R. (2011). *Acta Cryst. D* **67**, 282–292.
- Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D. & Bairoch, A. (2005). *The Proteomics Protocols Handbook*, edited by J. M. Walker, pp. 571–607. Totowa: Humana Press.
- Goldman, B. S., Lin, J. T. & Stewart, V. (1994). *J. Bacteriol.* **176**, 5077–5085.
- Kabsch, W. (2010). *Acta Cryst. D* **66**, 125–132.
- Lee, D. Y., Ramos, A., Macomber, L. & Shapleigh, J. P. (2002). *Appl. Environ. Microbiol.* **68**, 2140–2147.
- Lin, J. T. & Stewart, V. (1996). *J. Mol. Biol.* **256**, 423–435.
- McKellar, J. L., Minnell, J. J. & Gerth, M. L. (2015). *Mol. Microbiol.* **96**, 694–707.
- McPhillips, T. M., McPhillips, S. E., Chiu, H.-J., Cohen, A. E., Deacon, A. M., Ellis, P. J., Garman, E., Gonzalez, A., Sauter, N. K., Phizackerley, R. P., Soltis, S. M. & Kuhn, P. (2002). *J. Synchrotron Rad.* **9**, 401–406.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst. D* **67**, 355–367.
- Myrold, D. D. & Posavatz, N. R. (2007). *Soil Biol. Biochem.* **39**, 1737–1743.
- Parangan-Smith, A. & Lindow, S. (2013). *Appl. Environ. Microbiol.* **79**, 678–687.
- Shu, C. J., Ulrich, L. E. & Zhulin, I. B. (2003). *Trends Biochem. Sci.* **28**, 121–124.
- Upadhyay, A. A., Fleetwood, A. D., Adebali, O., Finn, R. D. & Zhulin, I. B. (2016). *PLoS Comput. Biol.* **12**, e1004862.
- Wadhams, G. H. & Armitage, J. P. (2004). *Nature Rev. Mol. Cell Biol.* **5**, 1024–1037.
- Wiley, J. M. & Waterbury, J. B. (1989). *Appl. Environ. Microbiol.* **55**, 1888–1894.
- Winn, M. D. *et al.* (2011). *Acta Cryst. D* **67**, 235–242.