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Crystal structure of a putative quorum sensing-regulated protein (PA3611) from the Pseudomonas-specific DUF4146 family

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen commonly found in humans and other organisms and is an important cause of infection, especially in patients with compromised immune defense mechanisms. The *PA3611* gene of *P. aeruginosa* PAO1 encodes a secreted protein of unknown function, which has been recently classified into a small Pseudomonas-specific protein family called DUF4146. As part of our effort to extend structural coverage of novel protein space and provide a structure-based functional insight into new protein families, we report the crystal structure of PA3611, the first structural representative of the DUF4146 protein family.

Keywords

Pseudomonas-specific protein family; DUF4146; Pfam PF13652; virulence factor; quorum-sensing; JCSG; structural genomics

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous environmental bacterium, which is found in soil, marshes and coastal marine habitats, as well as on plant and animal tissues. It is an opportunistic pathogen that is one of the top three causes of infection in humans^{1,2}. People afflicted with cystic fibrosis and compromised host defense mechanisms are at increased risk of infections from *P. aeruginosa*. *P. aeruginosa* PAO1 has a large 6.3 Mbp genome with 5,570 predicted open reading frames (ORFs)¹. As with other organisms, a substantial number of its genes lack functional characterization, although many of these have been assigned putative functional roles based on transcriptome profiling²⁻⁵ and structural genomics approaches⁶. The *PA3611* gene of *P. aeruginosa* PAO1 encodes a secreted protein of unknown function with a molecular weight of ~14 kDa (residues 1–136) and a calculated isoelectric point of 8.86. PSI-BLAST⁷ searches identify ~60 homologues of PA3611 (UniProt ID: Q9HY15), which are all domains of unknown function (DUF) found solely in different strains of *Pseudomonas*. These proteins have been recently classified into a small *Pseudomonas*-specific family in Pfam⁸, PF13652 (DUF4146), and are all secreted proteins of similar size comprising a single DUF4146 domain. An earlier proteomics analysis using 2D-PAGE and MALDI-TOF mass spectrometry revealed that PA3611 may be a Quorum Sensing (QS)-regulated protein and a potential virulence factor⁹. *P. aeruginosa* PAO1 has 195 known virulence factors according to the Virulence Factor Database¹⁰ and the *Pseudomonas* Genome Database¹¹ (<http://www.pseudomonas.com>). Here we report the crystal structure of PA3611 at 1.6 Å resolution, which was determined using the semi-automated, high-throughput pipeline of the Joint Center for Structural Genomics (JCSG), as part of the NIGMS Protein Structure Initiative (PSI). The structure provides the first structural representative of the PF13652 (DUF4146) protein family.

MATERIALS AND METHODS

Protein production and crystallization

Clones were generated using the Polymerase Incomplete Primer Extension (PIPE) cloning method¹². The gene encoding PA3611 (gi|15598807) was amplified by polymerase chain reaction (PCR) from *Pseudomonas aeruginosa* PAO1 genomic DNA using *PfuTurbo* DNA polymerase (Stratagene) and I-PIPE (Insert) primers (forward primer, 5'-ctgtacttccaggcGCCTCGCTCAAGGATTTTGAAGTGGAGC-3'; reverse primer, 5'-aattaagtcgcgttaCTTCTTGCCCTGGATGCGGCAGCTGCCG-3', target sequence in upper case) that included sequences for the predicted 5' and 3' ends. The expression vector, pSpeedET, which encodes an amino-terminal tobacco etch virus (TEV) protease-cleavable expression and purification tag (MGSDKIHNNHHHHENLYFQ/G), was PCR amplified with V-PIPE (Vector) primers (forward primer: 5'-taacgcgacttaattaactcgtttaacggtctccagc-3', reverse primer: 5'-gccctggaagtacaggtttctgtgatgatgatgatg-3'). V-PIPE and I-PIPE PCR products were mixed to anneal the amplified DNA fragments together. *Escherichia coli* GeneHogs (Invitrogen) competent cells were transformed with the I-PIPE / V-PIPE mixture and dispensed on selective LB-agar plates. The cloning junctions were confirmed by DNA sequencing. Using the PIPE method, the gene segment encoding residues Met1-Ala19 were deleted from the construct used for structure determination for expression of soluble protein

because it is predicted to contain a signal peptide based on SignalP¹³. Expression was performed in a selenomethionine-containing medium at 25°C. Selenomethionine was incorporated via inhibition of methionine biosynthesis¹⁴, which does not require a methionine auxotrophic strain. At the end of fermentation, lysozyme was added to the culture to a final concentration of 250 µg/ml, and the cells were harvested and frozen. After one freeze/thaw cycle, the cells were homogenized and sonicated in lysis buffer [50 mM HEPES pH 8.0, 50 mM NaCl, 10 mM imidazole, 1 mM Tris(2-carboxyethyl)phosphine-HCl (TCEP)] and the lysate was clarified by centrifugation at $32,500 \times g$ for 30 minutes. The soluble fraction was passed over nickel-chelating column (GE Healthcare) pre-equilibrated with lysis buffer, the column washed with wash buffer [50 mM HEPES pH 8.0, 300 mM NaCl, 40 mM imidazole, 10% (v/v) glycerol, 1 mM TCEP], and the protein was eluted with elution buffer [20 mM HEPES pH 8.0, 300 mM imidazole, 10% (v/v) glycerol, 1 mM TCEP]. The eluate was buffer exchanged with TEV buffer [20 mM HEPES pH 8.0, 200 mM NaCl, 40 mM imidazole, 1 mM TCEP] using a PD-10 column (GE Healthcare), and incubated with 1mg of TEV protease per 15 mg of eluted protein for 2 hr at ambient temperature followed by overnight at 4°C. The protease-treated eluate was passed over nickel-chelating column (GE Healthcare) pre-equilibrated with HEPES crystallization buffer [20 mM HEPES pH 8.0, 200 mM NaCl, 40 mM imidazole, 1 mM TCEP] and the column was washed with the same buffer. The flow-through and wash fractions were combined and concentrated to 10.5 mg/ml by centrifugal ultrafiltration (Millipore) for crystallization trials. PA3611 was crystallized using the nanodroplet vapor diffusion method¹⁵ with standard JCSG crystallization protocols¹⁶. Sitting drops composed of 200 nl protein solution mixed with 200 nl crystallization solution in a sitting drop format were equilibrated against a 50 µl reservoir at 277 K for 22 days prior to harvest. The crystallization reagent consisted of 2.0 M $(\text{NH}_4)_2\text{SO}_4$, 0.2 M Li_2SO_4 , and 0.1 M 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 10.5. Ethylene glycol was added to a final concentration of 15% (v/v) as a cryoprotectant. Initial screening for diffraction was carried out using the Stanford Automated Mounting system (SAM)¹⁷ at the Stanford Synchrotron Radiation Lightsource (SSRL, Menlo Park, CA). The diffraction data were indexed in orthorhombic space group $P2_12_12_1$. The oligomeric state of PA3611 in solution was determined to be monomeric using a $1 \times 30 \text{ cm}^2$ Superdex 200 size exclusion column (GE Healthcare)¹² coupled with miniDAWN (Wyatt Technology) static light scattering (SEC/SLS) and Optilab differential refractive index detectors (Wyatt Technology). The mobile phase consisted of 20 mM Tris pH 8.0, 150 mM NaCl, and 0.02% (w/v) sodium azide. The molecular weight was calculated using ASTRA 5.1.5 software (Wyatt Technology).

Data collection, structure solution and refinement

MAD data were collected at SSRL on beamline 9-2 at wavelengths corresponding to the high-energy remote (λ_1), inflection point (λ_2) and peak (λ_3) of a selenium MAD experiment using the BLU-ICE¹⁸ data collection environment. The data sets were collected at 100 K using a MarMosaic 325 CCD detector (Rayonix, USA). The MAD data were integrated and reduced using XDS¹⁹ and scaled with the program XSCALE. The heavy atom sub-structure and phasing calculations were performed using SOLVE²⁰. RESOLVE²¹ was used for density modification and ARP/wARP²² was used for automatic model building to 1.60 Å resolution. Model completion and crystallographic refinement were performed

with the λ_1 data set using COOT²³ and REFMAC5²⁴. The refinement protocol included the experimental phase restraints in the form of Hendrickson–Lattman coefficients from SOLVE and TLS refinement with one TLS group for the whole molecule. Data and refinement statistics are summarized in Table I^{25,26,27,28}

Validation and deposition

The quality of the crystal structure was analyzed using the JCSG Quality Control server (<http://smb.slac.stanford.edu/jcsg/QC>). This server verifies: the stereochemical quality of the model using AutoDepInputTool²⁹, MolProbity³⁰, and Phenix³¹, the agreement between the atomic model and the data using RESOLVE²¹, the protein sequence using CLUSTALW³², the ADP distribution using Phenix, and differences in $R_{\text{cryst}}/R_{\text{free}}$, expected $R_{\text{free}}/R_{\text{cryst}}$ and various other items including atom occupancies, consistency of NCS pairs, ligand interactions and special positions using in-house scripts to analyze refinement log file and PDB header. Protein quaternary structure analysis was performed using the PISA server³³. Figure 1B was adapted from an analysis using PDBsum³⁴ and other figures were prepared with PyMOL³⁵. Atomic coordinates and experimental structure factors for PA3611 to 1.60 Å resolution (PDB ID: 3npd) were deposited in the Protein Data Bank (www.wwpdb.org).

RESULTS AND DISCUSSION

Cloning, expression, purification and crystallization of PA3611 were carried out using standard Joint Center for Structural Genomics (JCSG; <http://www.jcsg.org>) protocols. N-terminal residues 1–19 were excluded from the expression construct due to the prediction of a signal peptide cleavage site. The crystal structure of PA3611 was determined by Multi-wavelength Anomalous Diffraction (MAD) phasing to a resolution of 1.60 Å. Data collection, model and refinement statistics are summarized in Table I^{25,26,27,28}. A single PA3611 molecule is present in the crystallographic asymmetric unit. The final model (Figure 1) includes Gly0 (left over after cleavage of the expression and purification tag), residues 20–131 of the 136 residues in the full-length protein, 2 sulfate ions and 4 CAPS molecules from the crystallization reagents, 2 1,2-ethane-diol molecules from the cryoprotectant, and 124 water molecules. The Matthews' coefficient³⁶ is 1.95 Å³/Da, with an estimated solvent content of ~37 %. The Ramachandran plot produced by MolProbity³⁰ shows that 100% of the residues are in the favored regions.

PA3611 is comprised of one structural domain with five β -strands (B1- B5) and five α -helices (H1-H5). Analysis of the crystallographic packing of PA3611 using the PISA server⁵ indicates that a monomer is the biologically relevant oligomeric state of the protein, consistent with the oligomeric state in solution from SEC. The β -strands form a twisted anti-parallel β -sheet flanked on one side by the helices. A disulfide bond is present between Cys92 and Cys130 in helices H3 and H5, respectively. A residue conservation analysis reveals that the conserved residues Ser38, Arg44, Ile46, Tyr55, Val83, Gln86, Ser90, Asn94, Arg98 and Tyr109 line a groove on the surface of the protein (Figure 2).

A search for other proteins of similar structure was carried out using DALI³⁷, SSM³⁸ and FATCAT³⁹ using default search parameters. The SSM (Secondary-Structure Match) search did not identify any significant match (the highest hit had Q-score of 0.14 and Z-score of

0.6), and the best hit with DALI was with fatty acyl-adenylate ligase/saframycin MX1 synthetase (PDB id 3lnv, 3.9 Å r.m.s.d., 10% sequence identity, 87 aligned Ca atoms, Z-score 5.0), although several other proteins gave hits with lower Z-scores. A search for similar structures using the flexible alignment mode in FATCAT resulted in several hits with significant scores (P-value < 0.05), most of which were to α - β class proteins. The most significant hit was to the C-terminal RNA-binding domain of *Escherichia coli* Era GTPase⁴⁰ (score of ~0.0006, r.m.s.d. of 2.7 Å, 5.6% sequence identity, alignment length of 92 Ca atoms, PDB id 1ega), which is involved in maturation of the 30S ribosome by binding to 16S ribosomal RNA⁴¹, and belongs to the alpha-lytic protease prodomain-like fold (SCOP fold 54805) and is a member of the prokaryotic type KH domain superfamily (KH-domain type II, SCOP 54814, Pfam clan CL0007) (Figure 3, 4). The KH domain has been shown to be involved in protein-protein interactions in addition to RNA binding⁴². Numerous lysine and arginine residues on a helix-turn-helix motif in the KH domain of Era (Arg239, Lys243, Lys244, Lys250, Lys253, Lys255, Arg262 and Lys263) are implicated in RNA interactions⁴⁰. Although these residues are not directly conserved in the equivalent positions in PA3611, there are some structural similarities and the basic nature of the helix is conserved: Era residues Arg239, Lys244, Lys255 and Lys263 are close to chemically similar residues Arg69, Arg75, Arg77, Arg84 and Arg98 in PA3611. In addition, PA3611 Arg131, which has no equivalent in Era, contributes to the basic nature of this region, which might be involved in ligand or nuclei acid interactions. Ile254 in Era is part of a hydrophobic core and is equivalent to Ile304 in the KH domain of the protein that is implicated in the fragile X syndrome link to mental retardation^{43,44}. In PA3611, Val83 is the corresponding residue and part of the conserved groove described above (Figure 2). Analysis of the electrostatic potential surface (using PDB2PQR⁴⁵ and the APBS⁴⁶ module in PyMOL) reveals an almost equal distribution of basic and acidic residues on the protein surface (Figure 5).

Analysis of potential interacting partners based on genomic context using STRING⁴⁷ (<http://string.embl.de>) indicates that PA3611 interacts with PA3612 (score ~0.8, a 73-residue protein of unknown function classified in PF12843, DUF3820). Also, PA3611 and PA3612 may form a single transcriptional unit based on the prediction that they form an operon according to the *Pseudomonas* Genome Database. It is also predicted to interact with its adjacent protein PotD (PA3610), which is the polyamine substrate-binding protein in the polyamine uptake system comprised of PotABCD. As polyamine transport has been implicated in quorum sensing and PA3611 was found to be up regulated in quorum sensing, PA3611 (and PA3612) may be involved in quorum sensing via modulation of PotD's function, with implications specific to biofilm formation in *Pseudomonas*. A computational assessment of PA3611 using a Support Vector Machine method as implemented in VirulentPred⁴⁸ (<http://203.92.44.117/virulent/index.html>) predicts PA3611 as a virulence factor. The structure of PA3611 provides some clues into the potential function of this protein and will serve as a guide for further investigation into its molecular and cellular role.

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Figure 1. Crystal structure of PA3611 from *Pseudomonas aeruginosa*

(A) Stereo ribbon diagram of PA3611 color-coded from N-terminus (blue) to C-terminus (red). Helices H1-H5, β -strands (B1-B5) and the Cys92-Cys130 disulfide bond are indicated. (B) Diagram showing the secondary structure elements of PA3611 superimposed on its primary sequence, adapted from PDBSum (<http://www.ebi.ac.uk/pdbsum>). The α -helices (H1-H5), β -strands (B1-B5), β -turns (β) and β -hairpins (red loops) are indicated. The sequence and structure includes Gly0, which remains after removal of the expression and purification tag, and residues 20–131 of the 136 residues in the entire protein.

Figure 2. Sequence conservation in PA3611

Residue conservation analysis performed using CONSURF⁴⁹ of PA3611(UniProt Q9HY15) homologs included in the analysis (with UniProt id codes and sequence identities to PA3611 in parentheses): PSPPH_3802 from *Pseudomonas syringae* pv. *phaseolicola* str. 1448A (Q48F97, 70%), PPUT_4084 from *Pseudomonas putida* str F1 (A5W7U8, 57%), PFL01_1180 from *Pseudomonas fluorescens* str Pf0-1 (Q3KH33, 59%), PFLU_1192 from *Pseudomonas fluorescens* str SBW25 (C3KDG3, 52%). The surface representation color gradient goes from cyan (most variable) to magenta (most conserved). Most of the conserved residues in PA3611 and its homologs are located on one side of the protein and are located on the surface or surround a prominent groove in the protein. They include Ser38, Arg44, Ile46, Tyr55, Val 83, Gln86, Ser90, Asn94, Arg98 and Tyr109 (labeled in left panel; orientation is similar to that in Fig. 1). Right panel: ~180° rotation around a vertical axis compared to the left panel.

Figure 3. Structural comparison of PA3611 with the RNA-binding domain of Era
 PA3611 (blue) is structurally similar to the C-terminal RNA-binding domain of *E. coli* Era GTPase (grey). Some of the Era residues (red sticks) implicated in RNA binding (Arg239, Lys244, Lys255 and Lys263) approximate the location of PA3611 residues (blue sticks) Arg69, Arg75, Arg77, Arg84 and Arg98, respectively. Although, only Arg98 is conserved in PA3611 homologs, the similar chemical nature of the residues leads to a similar basic region on both proteins. PA3611 Arg131, which is not a counterpart of any Era residues, also contributes to the basic nature of this region and might be involved in ligand or nucleic acid interactions.

Figure 4. Structural alignment of PA3611 Era

Superimposition of PA3611 (in blue to red from N- to C-terminus, similar orientation as in Figure 3) with the *E. coli* Era GTPase (grey) using DaliLite³⁷ (Z-score=3.2, r.m.s.d of 3.2 Å over 71 Cα residues) highlighting the significant structural differences in helix positions but the FATCAT flexible alignment mode is still able to identify the overall structural similarity between PA3611 and the C-terminal RNA-binding domain of Era.

Figure 5. Surface charge analysis

The electrostatic surface representation of PA3611 shows an almost equal distribution of positively- and negatively-charged residues (blue and red, respectively) on the protein surface. The basic region (blue) is primarily made up of residues described in Figure 3. The molecular orientation is similar to that in Figure 1. The color scale is in units of kT/e from -3 to +3.

TABLE I

Summary of crystal parameters, data collection and refinement statistics for PDB 3npd

Space group	P 2 ₁ 2 ₁ 2 ₁		
Unit cell parameters	a = 34.99 Å, b = 51.32 Å, c = 56.90 Å		
Data collection	λ ₁ MAD Se	λ ₂ MAD Se	λ ₃ MAD Se
Wavelength (Å)	0.91837	0.97941	0.97925
Resolution range (Å)	28.9–1.60	28.9–1.60	28.9–1.60
Highest resolution shell (Å)	1.64–1.60	1.64–1.60	1.64–1.60
Number of observations	49,327	49,052	49,176
Number of unique reflections	14,032	14,047	14,051
Completeness (%)	99.6 (99.3)	99.6 (99.4)	99.6 (98.9)
Mean I/σ (I)	10.9 (2.1)	11.1 (2.1)	10.5 (2.0)
R _{merge} on I [†] (%)	7.4 (64.9)	7.5 (62.6)	8.5 (64.8)
R _{meas} on I [‡] (%)	8.7 (76.6)	8.9 (73.8)	9.9 (76.4)
R _{p.i.m.} on I ^{‡‡} (%)	4.5 (40.0)	4.6 (38.5)	5.1 (39.8)
Model and refinement statistics			
Resolution range (Å)	28.9–1.60	Data set used in refinement	λ ₁
No. of reflections (total)	13,997 ^a	Cutoff criteria	F >0
No. of reflections (test)	700	R _{cryst}	0.160
Completeness (% total)	99.5	R _{free}	0.205
Stereochemical parameters			
Restraints (RMSD observed)			
Bond angle (°)	1.7		
Bond length (Å)	0.016		
Average isotropic B-value ^{‡‡} / Wilson plot B-value (Å ²)	22.7 / 17.8		
ESU based on R _{free}	0.096		
Protein residues/ atoms (Å)	113 / 868		
Water/ solvent molecules	124 / 8 (SO4=2, CAPS=4, EDO=2)		
Ramachandran plot: residues (%) in favored / allowed	100 / 100		

Values in parentheses are for the highest resolution shell.

^a Typically, the number of unique reflections used in refinement is slightly less than the total number that were integrated and scaled. Reflections are excluded due to systematic absences, negative intensities, and rounding errors in the resolution limits and cell parameters ESU = Estimated overall coordinate error ²⁶

$$^{\dagger}R_{merge} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

$$^{\ddagger}R_{meas} = \frac{\sum_{hkl} [N/(N-1)]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

$$^{\ddagger\ddagger}R_{p.i.m.} \text{ (precision-indicating } R_{merge}) = \frac{\sum_{hkl} [(1/(N-1))^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|]}{\sum_{hkl} \sum_i I_i(hkl)}.$$

$R_{cryst} = \frac{\sum |F_{obs}| - \sum |F_{calc}|}{\sum |F_{obs}|}$, where F_{calc} and F_{obs} are the calculated and observed structure-factor amplitudes, respectively. R_{free} is the same as R_{cryst} but for 5.0% of the total reflections chosen at random and omitted from refinement.

^{††} This value represents the total B that includes TLS and residual B components.