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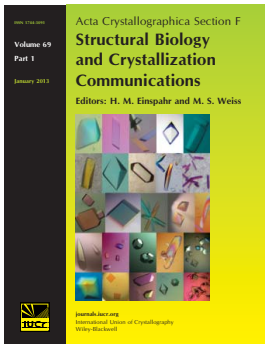

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# Crystallization and preliminary X-ray diffraction analysis of a DING protein from *Pseudomonas aeruginosa* PA14

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DING proteins form an emergent family of proteins consisting of an increasing number of homologues that have been identified in all kingdoms of life. They belong to the superfamily of phosphate-binding proteins and exhibit a high affinity for phosphate. In eukaryotes, DING proteins have been isolated by virtue of their implication in several diseases and biological processes. Some of them are potent inhibitors of HIV-1 replication/transcription, raising the question of their potential involvement in the human defence system. Recently, a protein from *Pseudomonas aeruginosa* strain PA14, named PA14DING or LapC, belonging to the DING family has been identified. The structure of PA14DING, combined with detailed biochemical characterization and comparative analysis with available DING protein structures, will be helpful in understanding the structural determinants implicated in the inhibition of HIV-1 by DING proteins. Here, the expression, purification and crystallization of PA14DING and the collection of X-ray data to 1.9 Å resolution are reported.

## 1. Introduction

In bacteria, phosphate uptake is mediated by two different systems: the phosphate inorganic transport system (Pit), which is used under high-phosphate conditions, and the phosphate-specific transport system (Pst), which is utilized during phosphate starvation (Willsky & Malamy, 1980). The latter involves an ATP-fueled ABC transporter, in which a periplasmic phosphate-binding protein (PBP or PstS) plays a phosphate-scavenging role (Wanner, 1993). In addition to PstS, which is encoded by the *pst* operon, some bacterial species encode other PBPs such as DING proteins (Berna, Scott *et al.*, 2009) or alkaline phosphatases (APs; Berna *et al.*, 2008), which share about 20–30% sequence identity with PstS. Interestingly, whereas PstS and AP proteins seem to be exclusively prokaryotic, DING proteins have been identified in all kingdoms of life (Berna, Scott *et al.*, 2009).

DING proteins were named according to their highly conserved N-terminal amino-acid sequence DINGGG– (Adams *et al.*, 2002). These proteins were initially identified in eukaryotes by virtue of their implication in numerous diseases and biological mechanisms (rheumatoid arthritis, nephrolithiasis, HIV inhibition and cell-cycle regulation; Adams *et al.*, 2002; Berna, Bernier *et al.*, 2009; Hain *et al.*, 1996; Kumar *et al.*, 2004; Darbinian *et al.*, 2009). However, functional studies of these proteins have been considerably hampered by a lack of genetic information, since neither a gene nor an ORF encoding DING proteins has been identified in eukaryotes (Berna, Scott *et al.*, 2009; Diemer *et al.*, 2008). The explosion of high-throughput genome sequencing in the last decade has revealed that DING proteins are widespread in *Pseudomonas* species. In contrast to eukaryotes, the encoding genes are well sequenced and properly annotated in these genomes and thus constitute a valuable resource for studies of DING proteins.

To date, only two structures of DING proteins have been solved: those of the human phosphate-binding protein (HPBP) isolated from human plasma (Morales *et al.*, 2006) and its homologue PfluDING from *P. fluorescens* SBW25 (Liebschner *et al.*, 2009; Moniot *et al.*, 2007). These proteins exhibit a fold consisting of two globular domains linked together by a flexible hinge (Ahn *et al.*, 2007). Their structures superimpose on those of PstS with the exception of four external protruding loops and two disulfide bridges that are present

in DING proteins but not in PstS (Berna, Bernier *et al.*, 2009; Liebschner *et al.*, 2009; Ahn *et al.*, 2007). The bound phosphate anion resides within a binding cleft formed by the two domains. Anion binding involves eight residues that form a complex network of 12 hydrogen bonds with the phosphate moiety (Liebschner *et al.*, 2009). Amongst these binding residues, an aspartic acid (Asp62) has been shown to play a critical role in discrimination between phosphate and other closely related ions such as sulfate or arsenate (Elias *et al.*, 2012; Liebschner *et al.*, 2009).

In pseudomonads, the gene encoding the DING protein is located in a conserved genomic region between a haemagglutinin-like gene and an Hxc type 2 secretion system. Recently, Ball *et al.* (2012) showed that the DING protein is indeed secreted *via* this machinery in clinical isolates that are closely related to the *P. aeruginosa* strain PA14. Despite their high affinity for phosphate, no clear role in phosphate uptake has been attributed to DING proteins in pseudomonads. However, these proteins are possibly involved, together with PstS, in the adherence of pathogenic *P. aeruginosa* to intestinal epithelial cells (Zaborina *et al.*, 2008). This adherence, which is required for the expression of cytotoxic effectors, is abolished in phosphate-rich media, suggesting regulation by the Pho regulon (Zaborina *et al.*, 2008).

In addition to their potential implication in bacterial virulence, some DING proteins isolated from humans (Lesner *et al.*, 2009; Cherrier *et al.*, 2011) and plants (Darbinian-Sarkissian *et al.*, 2006) show a potent ability to inhibit HIV-1 replication. The molecular mechanism by which viral inhibition occurs is still unknown, although DING proteins may specifically inhibit the transcriptional step of the viral cycle (Cherrier *et al.*, 2011). In this paper, we focus on the DING protein from *P. aeruginosa* strain PA14, which we named PA14DING (or LapC; Ball *et al.*, 2012). The resolution of its structure and the consequent structure–function studies will enable the mechanism of this inhibition to be deciphered. Here, we report the crystallization, data collection and primary crystallographic analysis of PA14DING.

## 2. Methods and materials

### 2.1. Cloning, expression and purification of the PA14DING protein

The PA14DING gene was amplified from the genomic DNA of *P. aeruginosa* strain PA14 (accession No. CP000438; locus tag PA14_55410) using the Gateway Cloning System (Invitrogen). Briefly, an initial PCR was performed to amplify the PA14DING gene (primers PA14DINGF, 5′-GGC AGC GGC GCG GAC ATC AAC GGC GGT GGC GCC ACC CTG CCG CAA CAG CTG TAC-3′, and PA14DINGR, 5′-GAA AGC TGG GTG TTA GAG CGG ACG GCC GAT GCC GTT GCA GAC GTT GGA ATG-3′). A second PCR step using primers containing *attB*1 and *attB*2 Gateway recombination sites (GatewayF, 5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC GAA AAC CTG TAT TTT CAG GGC AGC GGC GCG-3′, and GatewayR, 5′-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTG-3′) was performed to allow cloning into the Gateway pDONR221 vector (Moreland *et al.*, 2005). To allow the removal of fusion tags, a sequence encoding a tobacco etch virus (TEV) cleavage site was added downstream of the GatewayF primer. Finally, the PA14DING gene was subcloned into the destination vector pDEST-periHisMBP (Addgene plasmid 11086; Nallamsetty *et al.*, 2005), allowing the expression of an N-terminal hexa-His-MBP fusion tag for affinity purification of the protein. The resulting vector was transformed into *Escherichia coli* DH5$\alpha$ and extracted using a QIAprep Spin Miniprep Kit (Qiagen). The sequence of the gene was verified by sequencing.

PA14DING in fusion with His-MBP (His-MBP-PA14DING) was overexpressed in *E. coli* strain BL21(DE3)-pLysS. Protein expression was performed in 4 l auto-inducible ZYP medium (Studier, 2005; 100 µg ml$^{-1}$ ampicillin, 34 µg ml$^{-1}$ chloramphenicol; overnight culture at 310 K) inoculated with 100 ml overnight pre-culture. Cells were harvested by centrifugation (5000*g*, 15 min, 277 K), resuspended in lysis buffer (300 m*M* NaCl, 50 m*M* Tris pH 8, 1 m*M* PMSF, 0.25 mg ml$^{-1}$ lysozyme, 10 µg ml$^{-1}$ DNAse I, 20 m*M* $MgSO_4$) and stored at 193 K for 2 h. The frozen suspension was then thawed at 377 K and disrupted by three steps of sonication (Branson Sonifier 450; 30 s, 80% intensity and microtip limit of 8). The cell debris was pelleted by centrifugation (17 500*g*, 30 min, 277 K) and the supernatant was loaded onto a nickel-affinity column (GE Healthcare) at a flow rate of 5 ml min$^{-1}$. Elution of His-MBP-PA14DING was performed using a buffer consisting of 300 m*M* NaCl, 50 m*M* Tris pH 8, 250 m*M* imidazole. The eluted proteins were checked on a 15% SDS–PAGE gel. The gel revealed the presence of two major proteins migrating with mobilities of ∼80 and ∼40 kDa that could correspond to His-MBP-PA14DING and a partial expression product probably corresponding to His-MBP alone, respectively (data not shown).

```
ggcagcggcgcggacatcaacggcggtggcgccaccctgccgcaacagctgtaccaggagcccggcgtcctgaccgccggctttgccgcctacatcggcgtaggcagtggcaacggcaag
 g  s  g  a  D  I  N  G  G  G  A  T  L  P  Q  Q  L  Y  Q  E  P  G  V  L  T  A  G  F  A  A  Y  I  G  V  G  S  G  N  G  K
gcggccttcctgaacaacgactacaccaagttcgtcgccggcaccaccaacaagaacgtgcattgggctggtagcgactccaaactgagcaagaccaacgaaaccaaccccctatctgagc
 A  A  F  L  N  N  D  Y  T  K  F  V  A  G  T  T  N  K  N  V  H  W  A  G  S  D  S  K  L  S  K  T  N  E  T  N  P  Y  L  S
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 A  H  G  S  A  W  G  P  L  I  Q  V  P  S  V  A  T  S  V  A  L  P  F  N  K  S  G  S  N  A  V  N  F  A  D  V  N  T  L  C
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 G  V  F  S  G  R  L  T  D  W  S  Q  I  P  G  S  G  R  S  G  A  I  T  V  V  Y  R  S  E  S  S  G  T  T  E  L  F  T  R  F
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 L  N  A  S  C  S  S  T  L  E  G  G  T  F  A  I  T  T  S  F  G  S  S  F  S  G  G  L  P  A  G  A  V  S  A  Q  G  S  Q  A
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 V  M  N  A  L  N  A  A  Q  G  R  I  T  Y  M  S  P  D  F  A  A  P  T  L  A  G  L  D  D  A  T  K  V  A  Q  V  R  G  V  S
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 P  A  P  A  N  V  S  A  A  I  G  A  V  T  P  P  T  T  A  Q  R  S  D  P  N  N  W  V  P  V  F  A  A  T  A  N  P  N  D  P
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 S  V  R  P  Y  P  T  S  G  Y  P  I  L  G  F  T  N  L  I  F  S  Q  C  Y  A  N  A  T  Q  T  Q  Q  V  R  D  F  F  T  R  H
tacggcgccaccgccaacaacgacaccgcgatcaccaaccatcgcttcgtgccgctgccggcttcctggaagcttgccgtacgccagtcgttcctgacctccaccaacaacttgtacatc
 Y  G  A  T  A  N  N  D  T  A  I  T  N  H  R  F  V  P  L  P  A  S  W  K  L  A  V  R  Q  S  F  L  T  S  T  N  N  L  Y  I
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 G  H  S  N  V  C  N  G  I  G  R  P  L
```

**Figure 1**
Sequence of the PA14DING protein; the amino acids of the TEV recognition site that remain after cleavage are shown in lower case.

In order to separate these two proteins, a purification step was performed on a size-exclusion chromatography column (Superdex 75 16/60; GE Healthcare) in 20 m*M* Tris pH 8, 50 m*M* NaCl. Fractions containing His-MBP-PA14DING were pooled and incubated with 500 µl TEV protease (2.57 mg ml$^{-1}$) overnight at 277 K. After centrifugation (5000*g*, 10 min, 277 K), proteins were loaded onto a nickel-affinity column as described previously to eliminate His-MBP and potential remaining His-MBP-PA14DING. The flowthrough containing the PA14DING protein was then recovered and reloaded onto an anion-exchange column (HiTrap QFF GE Healthcare) to eliminate residual MBP, as the two proteins possessed similar molecular weights (∼43 kDa for MBP and ∼40 kDa for PA14DING). At pH 8 MBP is negatively charged and binds to the column, while the PA14DING protein, which is positively charged, can be recovered in the flowthrough. Finally, the PA14DING protein was dialyzed against a buffer consisting of 20 m*M* NaCl, 20 m*M* Tris pH 8 and concentrated to 10 mg ml$^{-1}$ (Amicon Ultra MWCO 10 kDa; Millipore, Ireland) prior to crystallization trials. The sequence of the crystallized protein is shown in Fig. 1.

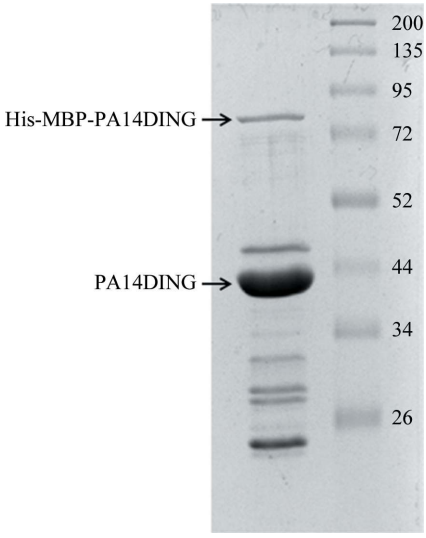


**Figure 2**
15% SDS–PAGE of PA14DING protein stained with Coomassie Blue. Right lane, molecular-weight markers (Thermo Scientific Spectra Multicolor broad-range protein ladder; labelled in kDa). Left lane, 5 µg PA14DING protein. PA14DING protein and the residual fusion protein His-MBP-PA14DING are indicated by arrows.

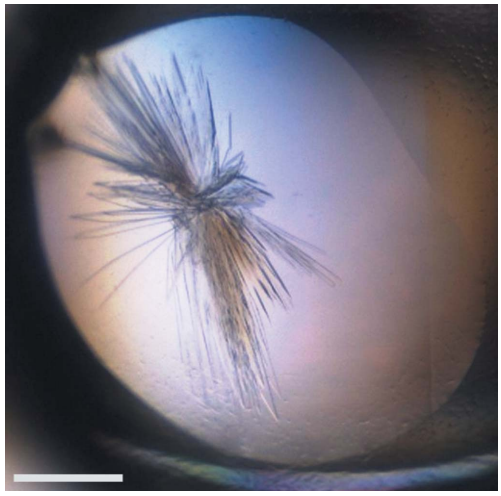

**Figure 3**
A typical cluster of plate-shaped PA14DING crystals; the scale bar (white) is 0.2 mm in length.

**Table 1**
Data-collection statistics.

Values in parentheses are for the last bin.

| | |
|---|---|
| Beamline | ID29, ESRF |
| Wavelength (Å) | 0.976 |
| Detector | PILATUS 6M |
| Oscillation (°) | 0.1 |
| No. of frames | 1800 |
| Resolution (Å) | 1.9 (2.0–1.9) |
| Space group | $P2_1$ |
| Unit-cell parameters (Å, °) | $a$ = 53.60, $b$ = 47.81, $c$ = 62.99, $\beta$ = 98.60 |
| No. of observed reflections | 80197 (11338) |
| No. of unique reflections | 24616 (3501) |
| Completeness (%) | 98.1 (98.6) |
| $R_{meas}$† (%) | 10.7 (43.9) |
| $\langle I/\sigma(I)\rangle$‡ | 12.86 (4.75) |
| Multiplicity | 3.26 (3.24) |
| Mosaicity (°) | 0.480 |

† $R_{meas}$ is the redundancy-independent merging $R$ factor: $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$. ‡ $I/\sigma(I)$ is the signal-to-noise ratio.

### 2.2. Protein crystallization

Crystallization assays were performed in 96-well trays incubated at 293 K using the sitting-drop vapour-diffusion method implemented on a nanodrop dispensing robot (Honeybee X8, Genomic Solutions). An initial round of screening (864 drops) was performed using the commercial crystallization screens Wizard I and II (Emerald BioSystems), Structure Screens I and II (Molecular Dimensions) and Stura Footprint Combination HT-96 (Molecular Dimensions). Despite the presence of impurities in this PA14DING preparation (Fig. 2), clusters of plate-shaped crystals appeared under various conditions. The best hit was identified as a condition from the Wizard I and II screens (Emerald BioSystems) containing 1.6 *M* ammonium sulfate. The pH and ammonium sulfate concentration of this condition were optimized (64 drops) using a commercial ammonium sulfate screen (AmSO4 Suite, Qiagen). The plate was incubated at 293 K and monitored using a Rock Imager and *Rock Maker* system (Formulatrix Inc., USA). Clusters of plates appeared after a few months at 293 K in a condition consisting of 100 m*M* MES pH 6.5, 2 *M* ammonium sulfate, 5%(*v*/*v*) PEG 400 (Fig. 3). Attempts to further optimize the crystals by using new screening strategies or by improving the best conditions using additives (Additive Screen HT, Hampton Research) yielded the same clusters of plates.

### 2.3. Data collection

A cryoprotectant solution consisting of the crystallization solution supplemented with 20%(*v*/*v*) glycerol was added to the drop (1 µl cryoprotectant in 300 nl drops) in order to gently exchange the solution containing the crystal. Next, the crystal was transferred into a drop containing 1 µl of the cryoprotective solution for 1 min prior to mounting on a MicroLoop (MiTeGen) and flash-cooling in liquid nitrogen. X-ray diffraction intensities were collected on the ID29 beamline at the ESRF (Grenoble, France) using a wavelength of 0.976 Å and a PILATUS 6M detector with 0.1 s exposures. Diffraction data were collected from 1800 images using the fine-slicing method; individual frames consisted of 0.1° steps over a range of 180° (Fig. 4).

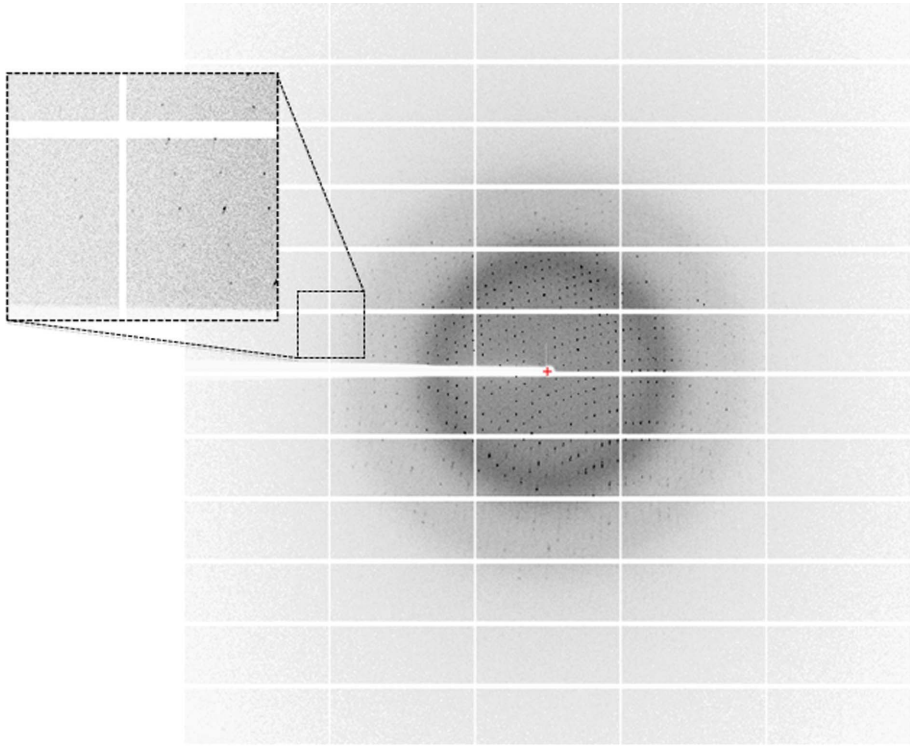

**Figure 4**
A diffraction pattern from a crystal of the PA14DING protein. The edge of the frame is at 1.30 Å resolution.

## 3. Results and conclusions

X-ray diffraction data were integrated and scaled using the *XDS* program (Kabsch, 2010; Table 1). The PA14DING crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 53.60$, $b = 47.81$, $c = 62.99$ Å, $\beta = 98.60^\circ$. The calculated Matthews coefficient $V_M$ suggests the presence of one monomer per asymmetric unit (with a $V_M$ of 2.1 Å$^3$ Da$^{-1}$, corresponding to a solvent content of 41.47%). Molecular replacement was performed with *Phaser* (McCoy *et al.*, 2007) using the structure of PfluDING as a model (74% sequence identity to PA14DING; PDB entry 2q9t; Ahn *et al.*, 2007), from which amino acids 231–238 were deleted. One molecule was placed in the asymmetric unit ($R_{free}$ = 33.29%) and the crystal packing was clearly complete. The solvent content of this crystal is low and is comparable to that observed for the high-resolution crystals of PfluDING ($V_M$ = 1.9 Å$^3$ Da$^{-1}$, corresponding to a solvent content of 37.9%; Moniot *et al.*, 2007). Manual model improvement was performed using *Coot* (Emsley & Cowtan, 2004) and refinement was performed with *REFMAC* (Murshudov *et al.*, 2011) and *PHENIX* (Adams *et al.*, 2010). The construction, refinement and interpretation of the structure are in progress.

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