Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

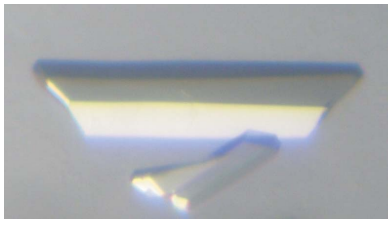
Expression, crystallization and preliminary X-ray crystallographic analysis of Xoo0352, D-alanine-D-alanine ligase A, from *Xanthomonas oryzae* pv. *oryzae*

Thanh Thi Ngoc Doan,[a] Jin-Kwang Kim,[a] Hyesoon Kim,[b] Yeh-Jin Ahn,[b] Jeong-Gu Kim,[c] Byoung-Moo Lee[c] and Lin-Woo Kang[a]*

[a]Department of Advanced Technology Fusion, Konkuk University, Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea, [b]Major in Life Science, College of Natural Sciences, Sangmyung University, 7 Hongji-dong, Jongno-gu, Seoul 110-743, Republic of Korea, and [c]Microbial Genetics Division, National Institute of Agricultural Biotechnology (NIAB), Rural Development Administration (RDA), Suwon 441-707, Republic of Korea

Correspondence e-mail: lkang@konkuk.ac.kr

Received 28 July 2008
Accepted 8 September 2008



Xanthomonas oryzae pv. *oryzae* (Xoo) causes bacterial blight (BB), which is one of the most devastating diseases of rice in most rice-growing countries. D-Alanine-D-alanine ligase A (DdlA), coded by the Xoo0352 gene, was expressed, purified and crystallized. DdlA is an enzyme that is involved in D-alanine metabolism and the biosynthesis of an essential bacterial peptidoglycan precursor, in which it catalyzes the formation of D-alanyl-D-alanine from two D-alanines, and is thus an attractive antibacterial drug target against Xoo. The DdlA crystals diffracted to 2.3 Å resolution and belonged to the primitive tetragonal space group $P4_32_12$, with unit-cell parameters $a = b = 83.0$, $c = 97.6$ Å. There is one molecule in the asymmetric unit, with a corresponding V_M of 1.88 Å^3 Da^{-1} and a solvent content of 34.6%. The initial structure was determined by molecular replacement using D-alanine-D-alanine ligase from *Staphylococcus aureus* (PDB code 2i87) as a template model.

1. Introduction

Rice (*Oryza sativa* L.) is an important staple for human consumption, especially in East, South and South East Asia. One of the most destructive diseases of rice, bacterial blight (BB), is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo; Ezuka & Kaku, 2000). In 2006, BB resulted in a production loss worth 100 million dollars in South Korea alone. There are currently no effective antibacterial drugs against Xoo. Recently, the complete genome sequence of Xoo has been determined (Lee *et al.*, 2005), providing valuable information for the selection of antibacterial drug-target proteins. As the first step towards developing antibacterial drugs against Xoo, we selected approximately 100 genes coding essential enzymes (Payne *et al.*, 2004, 2007) from the 4538 putative genes of Xoo (Lee *et al.*, 2005) as drug-target protein candidates. The genes coding these target proteins have been cloned and expressed systematically in *Escherichia coli*.

Peptidoglycan is an essential component of the bacterial cell wall, which is an attractive target for antibacterial drug development as similar structures and biosynthetic pathways are not found in plants or mammals (Spratt, 1994). In the peptidoglycan structure, D-alanine is one of the central molecules of the cross-linking step of peptidoglycan assembly, which increases the stability of the cell wall against proteolytic degradation. The D-alanine branch of peptidoglycan synthesis consists of three reactions with three catalysts: the pyridoxal phosphate-dependent D-alanine racemase (Alr), the ATP-dependent D-alanine-D-alanine ligase (Ddl) and the ATP-dependent D-alanyl-D-alanine-adding enzyme. Ddl catalyzes the formation of the precursor of peptidoglycan, D-alanyl-D-alanine dipeptide. The dimerization of D-alanine begins with an attack on the first D-alanine by the γ-phosphate of adenosine triphosphate (ATP) to yield an acylphosphate. This is followed by attack by the amino group of the second D-alanine, which eliminates the phosphate and yields the product D-alanyl-D-alanine (Walsh, 1989). To date, several antibiotics have been developed that inhibit bacterial cell-wall synthesis. The best known antibiotics are β-lactam antibiotics and vancomycin, which inhibit the cross-linking of cell-wall peptidoglycan precursors and hence lead to weakened cell walls as well as bacterial cell lysis

(Spratt, 1994; Groves *et al.*, 1994). However, extensive antibiotic usage and bacterial evolution have resulted in antibiotic resistance (Arthur *et al.*, 1996). Presently, D-cycloserine has been designed as an antibiotic targeting Ddl; however, its toxicity limits its usage (Feng & Barletta, 2003). Diazenedicarboxamides have been also developed as new inhibitors of D-alanine-D-alanine ligase and showed higher antibiotic effects than D-cycloserine, but their antimicrobial activity has only been tested in *E. coli* and *Staphylococcus aureus* (Kovac *et al.*, 2007). Therefore, new safe and wide-range antibiotics targeting Ddl are being developed.

This study describes the cloning, expression, purification, crystallization and preliminary X-ray crystallographic analysis of DdlA (Xoo0352). Three-dimensional structural studies of DdlA will elucidate the molecular basis of the enzymatic reaction mechanism of DdlA and will be useful for the design of a potential antibacterial drug against Xoo.

2. Methods and results

2.1. Cloning

The gene coding for DdlA was cloned from *X. oryzae* pv. *oryzae* (Xoo; KACC10331 strain) using the polymerase chain reaction (PCR). The sequences of the oligonucleotide primers were designed based on the published genome sequence (Lee *et al.*, 2005). The forward and reverse primers are 5′-GGG GGG **CAT ATG** CGC AAG ATC CGG GTC GGC CTC A-3′ and 5′-GGG GG**G GAT CC**T CAG TGT AGC TCG ACC GCG CTG C-3′, respectively. *Nde*I and *Bam*HI restriction sites are shown in bold. The PCR fragments were digested with *Nde*I and *Bam*HI and then ligated into modified pET11a vector previously digested with these enzymes. The modified vector contains six His residues and a tobacco etch virus (TEV) protease cleavage site before the *Nde*I site in the pET11a vector (Novagen) to facilitate purification of the expressed protein.

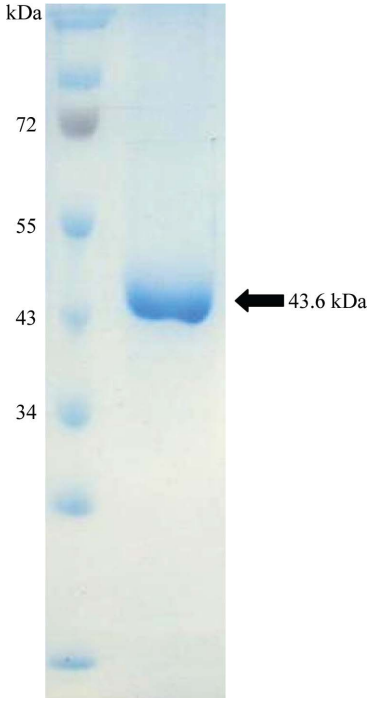


Figure 1
Purified DdlA is shown on a 10% SDS–PAGE gel.

2.2. Overexpression and purification

Expression of DdlA was performed in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin using *E. coli* strain BL21 (DE3) pLysS. Induction was carried out by adding 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside to the culture at 310 K when the the OD_{600} reached 0.6. After induction, the cells were cultured at 288 K for an additional 20 h. Cultured cells were harvested by centrifugation for 10 min at 6000 rev min^{-1} (Vision VS24-SMTi V5006A rotor) at 277 K. Cell pellets were then resuspended in ice-cold lysis buffer (25 m*M* Tris–HCl pH 7.5, 300 m*M* NaCl, 15 m*M* imidazole, 3 m*M* *β*-mercaptoethanol) and homogenized using ultrasonication on ice (Sonomasher, S&T Science, Korea). The lysate was centrifuged for 30 min at 13 000 rev min^{-1} (Vision VS24-SMTi V508A rotor) at 277 K. About 25% of the total expressed DdlA was soluble (data not shown). The supernatant containing soluble DdlA was loaded onto Ni–NTA His-Bind resin (Novagen) previously equilibrated with lysis buffer. Affinity purification was performed according to the manufacturer's protocol at 277 K. Lysis buffer containing 30 m*M* imidazole was used to wash out nonspecifically bound proteins. Lysis buffer containing 200 m*M* imidazole was applied to elute the 6×His-tagged DdlA protein. The resulting protein solution was dialyzed for 12 h at 277 K in buffer *A* (25 m*M* Tris–HCl pH 7.5, 3 m*M* *β*-mercaptoethanol) and further purified on an UNO6 Q column (Bio-Rad) using buffer *B* (25 m*M* Tris–HCl pH 7.5, 1 *M* NaCl, 3 m*M* *β*-mercaptoethanol) as an elution reagent. The homogeneity of the purified protein was examined using SDS–PAGE during each step. Only one band was visible on the SDS–PAGE gel after the two-step purification and it showed that the purified protein has a molecular weight of about 43.6 kDa, which is in agreement with the predicted molecular

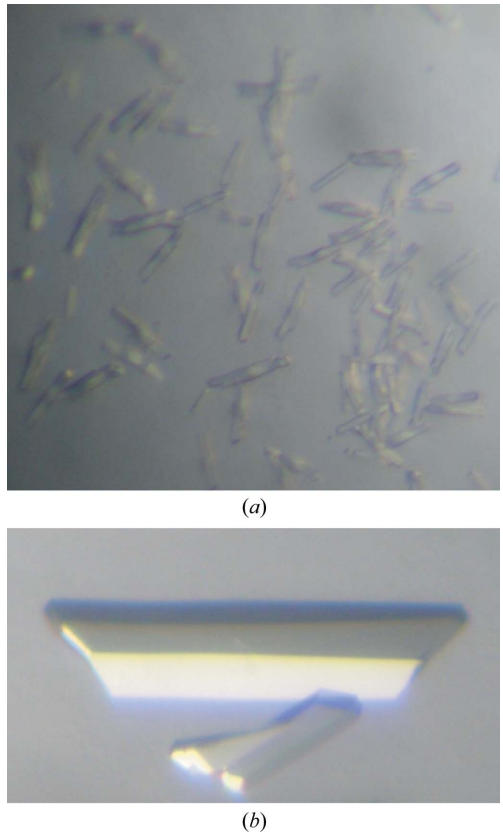


Figure 2
(*a*) Multiple crystals grew in the inital crystallization conditions. (*b*) A crystal of DdlA grew to a largest dimension of 0.4 mm in 1 d after optimization.

Table 1
Data-collection statistics.

Values in parentheses are for the outer shell.

X-ray source	17A, Photon Factory
Space group	$P4_32_12$
Unit-cell parameters (Å)	$a = b = 83.0$, $c = 97.6$
Wavelength	1.00000
Resolution (Å)	50.0–2.3 (2.4–2.3)
No. of observed reflections	136753
No. of unique reflections	16812
Redundancy	8.2 (8.3)
Completeness (%)	99.9 (100.0)
$R_{\rm merge}$† (%)	6.3 (26.9)
$I/\sigma(I)$	48.3 (10.5)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

weight of 39.6 kDa with an additional 4 kDa affinity tag (Fig. 1). The protein was then concentrated to 6 mg ml^{-1} in lysis buffer containing 25 m*M* NaCl using a Centri-Prep concentrating device with 30 000 Da molecular-weight cutoff (Millipore).

2.3. Crystallization and X-ray diffraction data collection

Crystallization conditions were initially screened at 287 K using a Hydra II e-drop automated pipetting system (Matrix) in 96-well Intelliplates (Art Robbins) using the Crystal Screen HT and Index Screen HT screening kits (Hampton Research). We obtained several different crystallization conditions from these two screens and Crystal Screen HT condition A6 [30%(*w*/*v*) PEG 4000, 0.1 *M* Tris pH 8.0, 0.2 *M* $MgCl_2$] was chosen as the best condition for crystal growth. This condition was optimized by varying the concentration of the precipitant PEG and the pH of the buffer while maintaining a protein concentration of 6 mg ml^{-1}. This optimization process was carried out using the sitting-drop vapour-diffusion method, in which droplets composed of a mixture of 1 µl protein solution and 1 µl reservoir solution were placed in vapour equilibration against a reagent reservoir. Multiple small crystals grew in all of the drops. Additive screening was tried in order to improve the quality of crystals. For additive screening, drops were set up as follows: 2.5 µl protein solution plus 2.0 µl reservoir solution plus 0.5 µl additive screen (Hampton Research Additive Screen, HR2-428). Diffraction-quality crystals were obtained at 287 K from a reservoir containing 15%(*w*/*v*) PEG 4000, 0.1 *M* Tris pH 8.5, 0.2 *M* $MgCl_2$ with 0.3 *M* dimethylethyl-(3-sulfopropyl)-ammonium as an additive. A crystal (longest edge 0.4 mm) which grew from this condition in 24 h is shown in Fig. 2.

The crystals were flash-cooled in liquid nitrogen using a cryoprotectant consisting of the reservoir solution plus 20%(*v*/*v*) glycerol. X-ray diffraction data were collected to 2.3 Å resolution from the frozen crystals using an ADSC Quantum 270 CCD detector on beamline 17A of the Photon Factory, High Energy Accelerator Research Organization, Japan. The diffraction data were collected using 1° oscillations with a crystal-to-detector distance of 169.85 mm. The data were integrated and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystallographic space group $P4_32_12$ was derived by auto-indexing (Otwinowski & Minor, 1997), with unit-cell parameters $a = b = 83.0$, $c = 97.6$ Å. Data-collection statistics are provided in Table 1. According to the Matthews coefficient calculation (Matthews, 1968), there is one molecule in the asymmetric unit, with a solvent content of 34.6%. The structure was determined by molecular replacement using *Phaser* from the *CCP*4 program package (McCoy *et al.*, 2005) with a homologue model generated using D-alanine-D-alanine ligase from *S. aureus* (PDB code 2i87, Liu *et al.*, 2006; sequence identity 38.0%) as a template model. The initial *R* factor from the molecular-replacement search was 46.5% and the resulting electron-density maps are of high quality. Further refinement and model building are in progress. The structural details will be described in a separate paper. Our structural data for DdlA will provide an insight into its enzymatic mechanisms and will be useful for developing antibacterial drugs against Xoo.

We are grateful to the staff members at beamline 17A of Photon Factory (KEK), Japan and to Seok-Joon Ahn, Seoul National University, Republic of Korea for his technical assistance. This work was supported by a grant (Code No. 20070501034003) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

References

Arthur, M., Reynolds, P. & Courvalin, P. (1996). *Trends Microbiol.* **4**, 401–407.
Ezuka, A. & Kaku, H. (2000). *Bull. Natl Inst. Agrobiol. Resour. (Jpn)*, **15**, 53–54.
Feng, Z. & Barletta, R. G. (2003). *Antimicrob. Agents Chemother.* **47**, 283–291.
Groves, P., Searle, M. S., Mackay, J. P. & Williams, D. H. (1994). *Structure*, **2**, 747–754.
Kovac, A., Majce, V., Lenarsic, R., Bombek, S., Bostock, J. M., Chopra, I., Polanc, S. & Gobec, S. (2007). *Bioorg. Med. Chem. Lett.* **17**, 2047–2054.
Lee, B. M. *et al.* (2005). *Nucleic Acids Res.* **33**, 577–586.
Liu, S., Chang, J. S., Herberg, J. T., Horng, M. M., Tomich, P. K., Lin, A. H. & Marotti, K. R. (2006). *Proc. Natl Acad. Sci. USA*, **103**, 15178–15183.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
McCoy, A. J., Grosse-Kunstleve, R. W., Storoni, L. C. & Read, R. J. (2005). *Acta Cryst.* D**61**, 458–464.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Payne, D. J., Gwynn, M. N., Holmes, D. J. & Pompliano, D. L. (2007). *Nature Rev. Drug Discov.* **6**, 29–40.
Payne, D. J., Gwynn, M. N., Holmes, D. J. & Rosenberg, M. (2004). *Methods Mol. Biol.* **266**, 231–259.
Spratt, B. G. (1994). *Science*, **264**, 388–393.
Walsh, C. T. (1989). *J. Biol. Chem.* **264**, 2393–2396.