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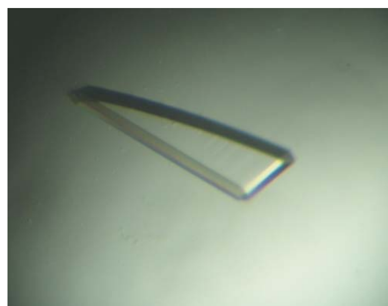

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Cloning, expression, crystallization and preliminary X-ray crystallographic analysis of malonyl-CoA–acyl carrier protein transacylase (FabD) from *Xanthomonas oryzae* pv. *oryzae*

Xanthomonas oryzae pv. *oryzae* (Xoo) causes bacterial blight in rice, which is one of the most devastating diseases in rice-cultivating countries. The *Xoo0880* (*fabD*) gene coding for a malonyl-CoA–acyl carrier protein transacylase (MCAT) from Xoo was cloned and expressed in *Escherichia coli*. MCAT is an essential enzyme that catalyzes a key reaction of fatty-acid synthesis in bacteria and plants: the conversion of malonyl-CoA to malonyl-acyl carrier protein. The FabD enzyme was purified and crystallized in order to elucidate its three-dimensional structure and to determine its enzymatic reaction mechanism and biological importance. The crystal obtained diffracted to 1.9 Å resolution and belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 41.4$, $b = 74.6$, $c = 98.5$ Å. According to Matthews coefficient calculations, the crystallographic structure contains only one monomeric unit in the asymmetric unit with a V_M of 2.21 $Å^3$ Da^{-1} and a solvent content of 44.3%.

1. Introduction

Rice is an important staple for human consumption all over the world. Bacterial blight (BB) is a highly destructive bacterial disease of rice that is caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo; Ezuka & Kaku, 2000). According to a report of the Agricultural Department, BB resulted in a rice-production loss worth more than 100 million dollars in South Korea in 2006 alone. To date, no effective drugs have been developed against this disease to halt rice-production losses. The whole genomic sequences of Xoo has been determined recently (Lee *et al.*, 2005) and provides valuable information for the selection of drug-target proteins from Xoo. As an initial step in drug development against Xoo, hundreds of genes coding essential enzymes have been selected as target proteins for drug candidates (Payne *et al.*, 2004, 2007) from the 4538 putative genes (Lee *et al.*, 2005). The selected target genes have been cloned and expressed in *Escherichia coli* systematically in order to obtain the essential drug-target enzymes and to determine their three-dimensional structures *via* X-ray crystallography.

Xoo0880 (FabD) is one of the most important drug-target enzymes of Xoo. The *fabD* gene expresses malonyl-CoA–acyl carrier protein transacylase (MCAT), which transfers the malonyl moiety to holo acyl carrier protein (ACP), forming a malonyl-ACP intermediate, in type II fatty-acid synthesis in bacteria (Ruch & Vagelos, 1973). The malonyl-ACP intermediate then acts as a two-carbon donor in the elongation step of fatty-acid synthesis. Fatty-acid synthesis (FAS) is essential for the survival of an organism (Magnuson *et al.*, 1993; White *et al.*, 2005) and MCAT takes part in this by extending the length of the growing acyl chain by two C atoms. MCAT has also been reported to be involved in polyketide biosynthesis, producing one of the largest classes of secondary metabolites, which includes tetracyclines and erythromycins (Keatinge-Clay *et al.*, 2003; Summers *et al.*, 1995). Therefore, MCAT is considered to be a vital enzyme in bacterial metabolic activity.

Here, we report the cloning and expression of the *fabD* (*Xoo0880*) gene and the purification, crystallization and preliminary X-ray crystallographic analysis of the coded enzyme. Crystallographic

three-dimensional structural studies of FabD will elucidate the molecular basis of its enzymatic reaction mechanism as an MCAT and be helpful in the design of a potential drug against Xoo.

2. Methods and results

2.1. Cloning

The FabD-coding sequence of *Xoo0880* was amplified *via* the polymerase chain reaction (PCR) using bacterial cells (Xoo KACC10331 strain) as a template. The sequences of the forward and reverse oligonucleotide primers, designed based on the published genome sequence (Lee *et al.*, 2005), were as follows: 5′-GGG GGG **CAT ATG** ACC GAA TCC ACT CTC GCC TTC A-3′ and 5′-GGG GGG **GGA T CC** TCA GTG GCC CCA CGC GTC GAG A-3′. The bases in bold designate *Nde*I and *Bam*HI restriction sites. The amplicon was double-digested with the two DNA-restriction enzymes and inserted into a modified pET11a vector (His-TEV-pET11a), which was engineered to contain additional residues for a 6×His tag and a tobacco etch virus (TEV) protease cleavage site before the *Nde*I site in a pET11a vector (Novagen) to facilitate purification of the expressed protein.

2.2. Overexpression and purification

The His-TEV-pET11a expression vector containing the coding sequence for Xoo0880 was introduced into *Escherichia coli* BL21 (DE3) cells. Cells were grown at 310 K to an OD_{600} of 0.5 in Luria–Bertani (LB) medium containing 50 $\mu g\ ml^{-1}$ ampicillin. Protein expression was induced by the addition of 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were cultured at the same temperature for an additional 16 h. Cultured cells were harvested by centrifugation for 20 min at 6000 rev min^{-1} (Vision VS24-SMTi V5006A rotor) at 277 K. The cell pellet was then resuspended in ice-cold lysis buffer (25 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 10 m*M* imidazole) and homogenized using ultrasonication (Sonomasher, S&T Science, Korea). The lysate was centrifuged for 30 min at 12 000 rev min^{-1} (Vision VS24-SMTi V508A rotor) at 277 K. Only 10% of the total expressed Xoo0880 was soluble (data not shown). The supernatant containing soluble FabD protein was applied onto Ni–NTA resin (Novagen) and affinity purification was performed according to the manufacturer's protocol at 277 K. The 6×His-tagged FabD protein was then eluted using lysis buffer containing 200 m*M* imidazole and treated with TEV protease overnight at 277 K to remove the 6×His tag. The resulting protein solution was dialyzed for 6 h at 277 K in buffer *A* (25 m*M* Tris–HCl pH 7.5, 15 m*M* NaCl) and

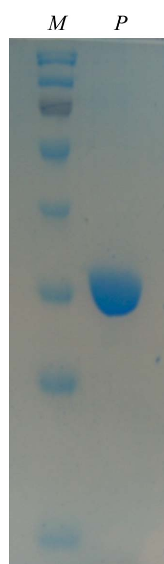
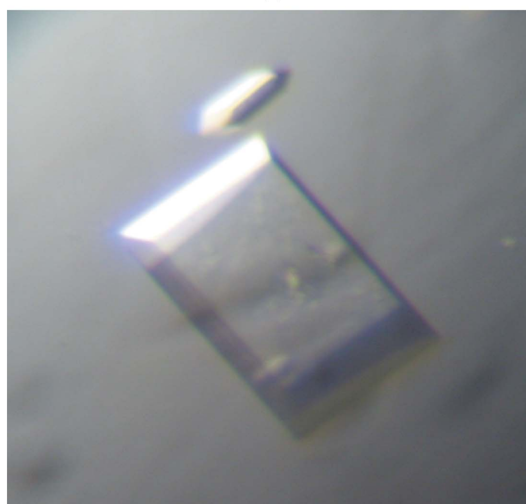
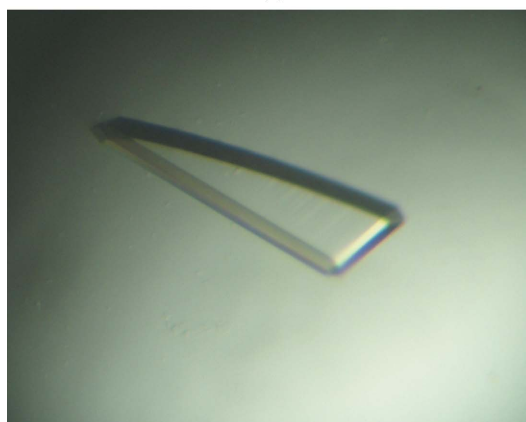


Figure 1
SDS–PAGE analysis of FabD (Xoo0880) during purification. Proteins were analyzed on 12% SDS–PAGE and stained with Coomassie Blue. Lane *M*, molecular-weight markers (kDa); lane *P*, purified FabD after UNO 6Q column chromatography.

(*a*)



(*b*)



(*c*)

Figure 2
Crystals of the FabD enzyme from *X. oryzae* pv. *oryzae* (Xoo) obtained from (*a*) a sitting-drop setup (Wizard G-5) using a Hydra automated high-throughput crystal screening machine, (*b*) a hanging-drop vapour-diffusion setup using 1.5 *M* ammonium sulfate, 0.1 *M* CHES pH 9.3, 0.2 *M* NaCl and (*c*) a well diffracting triangular shaped crystal grown at pH 9.0.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell.

Synchrotron	PF 17A
Wavelength (Å)	0.96418
Resolution range (Å)	50.0–1.9
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	
a	41.4
b	74.6
c	98.5
Total No. of reflections	62374
No. of unique reflections	18355
Completeness (%)	74.9 (32.7)
Molecules per ASU	1
V_M ($Å^3$ Da^{-1})	2.21
Solvent content (%)	44.3
Average $I/\sigma(I)$	30.9 (3.7)
R_{merge}† (%)	6.0 (33.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation of reflection *hkl* and $\langle I(hkl)\rangle$ is the average intensity of reflection *hkl*.

further purification was carried out on an UNO 6Q column (BioRad). The homogeneity of the purified protein was assessed *via* SDS–PAGE (Fig. 1). The purified protein was concentrated to 7 mg ml^{-1} and used in the crystallization process.

2.3. Crystallization and X-ray diffraction (data collection)

Crystallization of this enzyme was initiated by high-throughput crystal screening at 283 K using a Hydra II e-drop automated pipetting system (Matrix) with 96-well Intelli-Plates (Art Robbins) and various screening kits such as Crystal Screen HT, Index HT and Salt Rx HT (Hampton Research). Initially, very thin needle-like crystals (Fig. 2*a*) were obtained using Wizard condition G-5 (0.1 *M* CHES pH 9.5, 1.26 *M* ammonium sulfate and 0.2 *M* sodium chloride). Crystals were reproduced using the hanging-drop method, with a drop made up of 1 µl protein solution mixed with 1 µl reservoir solution and equilibrated against 1 ml reservoir solution; crystals grew over a period of a week. Since the initial crystals were not good enough for X-ray diffraction, the crystallization conditions were optimized by changing the pH and the salt concentration. Finally, a well diffracting crystal (Fig. 2*c*) was obtained in a week using 0.1 *M* CHES pH 9.0, 1.5 *M* ammonium sulfate and 0.2 *M* sodium chloride. The crystal was frozen in liquid nitrogen with 25%(*v*/*v*) glycerol as a cryoprotectant and used for data collection. The crystal data were collected using an ADSC Quantum 270 CCD detector on beamline 17A at the Photon Factory (KEK), Japan. X-ray diffraction data were collected to 1.9 Å resolution and each data set was integrated and scaled using *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997). The crystallographic space group $P2_12_12_1$ was derived for the crystal by auto-indexing (Otwinowski & Minor, 1997), with unit-cell parameters *a* = 41.4, *b* = 74.6, *c* = 98.5 Å. Data-collection statistics are provided in Table 1. According to the Matthews coefficient calculation (Matthews, 1968), only one crystallographic molecule was present in the asymmetric unit, with a solvent content of 44.3%. Structure determination was initiated by molecular replacement (MR) using *MOLREP* (Vagin & Teplyakov, 1997) with the structure of malonyl-CoA–acyl carrier protein transacylase (PDB code 1mla, Serre *et al.*, 1995) from *E. coli* as a template and the solution indicated that only one crystallographic molecule was present in the asymmetric unit. The template structure shared about 54.2% sequence identity with the Xoo FabD enzyme. The output model structure of this MR solution shows a well fitted electron-density map ($2F_o - F_c$) for all residues and the model will be improved by refinement. The structural details will be described in a separate paper. The structural data of the FabD (Xoo0880) protein will provide an insight into its enzymatic mechanism and be useful for developing antibacterial drugs against Xoo.

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