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Overexpression, Crystallization, and Preliminary X-Ray Crystallographic Analysis of the Alanine Racemase from *Enterococcus faecalis* v583

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Alanine racemase, a bacterial enzyme belonging to the fold-type III group of pyridoxal 5'-phosphate (PLP)-dependent enzymes, has been shown to catalyze the interconversion between L- and D-alanine. The alanine racemase from the pathogenic bacterium *Enterococcus faecalis* v583 has been overexpressed in *E. coli* and was shown to crystallize an enzyme at 295 K, using polyethylene glycol (PEG) 8000 as a precipitant. X-ray diffraction data to 2.5 Å has been collected using synchrotron radiation. The crystal is a member of the orthorhombic space group, $C222_1$, with unit cell parameter of a=94.634, b=156.516, c=147.878 Å, and $\alpha=\beta=\gamma=90^{\circ}$. Two or three monomers are likely to be present in the asymmetric unit, with a corresponding V_m of 3.38 $Å^3$ Da^{-1} and 2.26 $Å^3$ Da^{-1} and a solvent content of 63.7% and 45.5%, respectively.

Keywords: Alanine racemase, PLP, *Enterococcus faecalis*, preliminary X-ray analysis

The genus *Enterococcus* includes more than 20 species. Among them, *Enterococcus faecalis* is the species that most commonly causes human disease. Enterococci are naturally resistant to the penicillins, and have also been shown to become resistant to other drug classes *via* the acquisition of foreign DNA [9]. *Enterococcus* spp. rank third among the most common UTI (urinary tract infections) pathogens, and are also a common cause of chronic or recurrent UTIs, particularly those associated with structural abnormalities and instrumentation [8]. Despite the role of *E. faecalis* as a leading cause of nosocomial UTIs, little remains known regarding the bacterial factors involved in such infections. *E. faecalis* may bind to and activate bladder epithelial cells, setting the stage for secondary, more symptomatic infections. Despite the current recognition of *E. faecalis* as an important uropathogen, much remains to be determined regarding the pathogenicity of this infection [8]. Enterococci have become a significant cause of nosocomial infections in recent years. Bonten *et al.* [1] reported that *E. faecalis* plays an etiological role in colonization rates and polymicrobial pneumonia, and their study also indicated that it seldom causes respiratory tract infections. *E. faecalis* colonization and infection have been assumed to originate from endogenous sources [1]. However, previous studies have demonstrated that the colonization of the upper respiratory tract by *E. faecalis* is frequently preceded by gastric colonization, thereby suggesting that colonization occurred *via* the so-called gastropulmonary route [1].

Alanine racemase (AlaR, E.C. 5.1.1.1) is a member of the fold-type III group of pyridoxal 5'-phosphate (PLP)-dependent bacterial enzymes, which catalyzes the interconversion of L- and D-alanine, essential constituents of the peptidoglycan layer of the bacterial cell wall [10]. The enzyme is universal to bacteria, including mycobacteria, making it an attractive target for drug design. All known bacteria require D-Ala, whereas only L-alanine is utilized in protein synthesis in mammals. Presumably, specific AlaR inhibitors would kill bacteria but would exert no adverse side effects, as no known mammalian AlaRs exist. AlaR's catalytic activity depends on the binding of the PLP cofactor, a phosphorylated and oxidized form of vitamin B_6. PLP binds covalently to the protein *via* an imine bond to the ε-amino group of the side chain of a lysine, which is commonly designated as the internal aldimine (Schiff's base) form [5]. In addition, AlaR is the essential constituent of the peptidoglycan layer in both Gram-negative and Gram-positive bacteria, and requires pyridoxal 5'-phosphate (PLP) as a cofactor attached to the enzyme *via* an internal Schiff's base linkage. All known bacteria require D-alanine for peptidoglycan biosynthesis, whereas only L-alanine is employed in the synthesis of

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eukaryotic proteins [4]. The reaction is initiated *via* Schiff base formation between the substrate and cofactor (a common step among all PLP-dependent transformations), following which the substrate R-carbon can be deprotonated and stabilized by electron delocalization *via* the electrophilic pyridinium nitrogen. In either the natural D- or synthetic L-isomer forms, cycloserine functions as an essentially irreversible inhibitor of many PLP-dependent enzymes, including alanine racemase [2]. Cycloserine functions as a suicide inhibitor of alanine racemase and, as such, serves as an antimicrobial agent. The L-isomers of amino acids are frequently found in nature and generated *via* a variety of biosynthetic pathways. However, formation of their enantiomeric mates normally requires some form of chiral reduction, or an enzymatically catalyzed equilibration about the R-carbon between the two isomers [2].

Overall, the alanine racemase enzyme occurs ubiquitously in eubacteria and is indispensable, because D-alanine is an essential component of the peptidoglycans in eubacterial cell walls. Therefore, the enzyme is an attractive target for the development of mechanism-based inactivators, which may prove useful as antibacterial agents [10]. In order to provide further structural data on this important class of antibacterial target, we have started to determine the structure of AlaR from *E. faecalis* (EFAlaR). It is composed of 371 amino acids residues (M_r=40,980 Da). For this study, we overexpressed, purified, and crystallized EFAlaR and conducted a preliminary X-ray crystallographic analysis of EFAlaR. The structural information acquired in this study may provide insights into the design of drugs for the development of new antibiotics.

The full-length AlaR gene was amplified *via* the polymerase chain reaction using the *E. faecalis v583* genomic DNA as a template. The sequences of the forward and reverse oligonucleotide primers designed from the published genome sequence [8] were as follows: 5'-G GAA TTC **CAT ATG** GTC GTT GGA TGG CAT CGT C-3' and 5'-CCG CCG **CTC GAG** GTT ATA TTC TCT GGG GAT TCT TTG-3', respectively. The bases in bold designate the NdeI and XhoI digestion sites. The amplified DNA was then inserted into the NdeI/XhoI-digested expression vector, pET-22b (Novagen). EFAlaR was overexpressed in *E. coli* BL21 (DE3) cells. The cells were grown at 310 K to an OD_{600} of 0.6 in Luria-Bertani medium containing 50 μg/ml ampicillin. Protein expression was induced *via* the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cell growth was continued at 295 K overnight after IPTG induction, and the cells were harvested *via* 20 min of centrifugation at 4,200 ×*g* at 277 K. The cell pellet was then resuspended in ice-cold lysis buffer [50 mM Tris-HCl (pH 8.5), 200 mM NaCl, 5 mM imidazole, 2.5% (v/v) glycerol] and homogenized with a microfluidizer (Microfludics). The crude cell extract was centrifuged for 30 min at 12,000 rpm (Hanil Supra 21 K rotor) at 277 K.

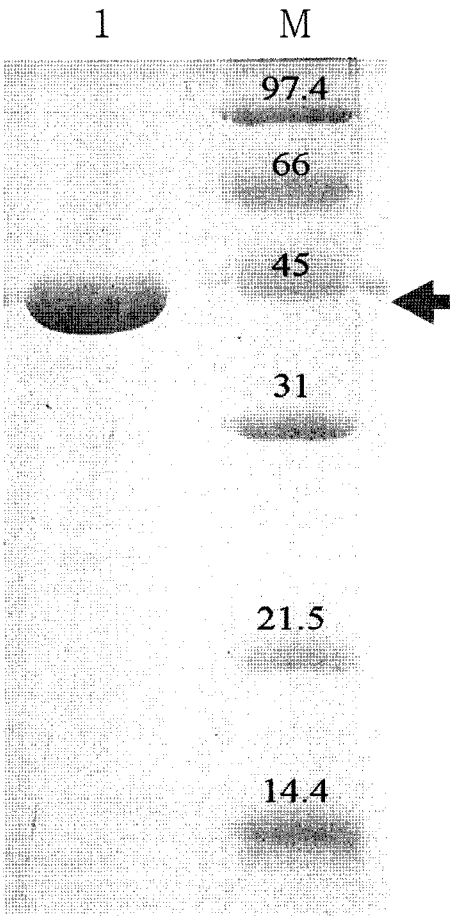

Fig. 1. SDS-PAGE [15% (v/v)] of the recombinant protein from *Enterococcus faecalis*.
Lane 1, the purified alanine racemase from *Enterococcus faecalis* after gel filtration; Lane M, molecular mass markers (kDa).

The recombinant protein in the supernatant fraction was purified *via* three chromatographic steps. The first step employed the C-terminal histidine tag by metal-chelate chromatography on Ni^{2+}-NTA resin (Qiagen). Later, ion-exchange chromatography was conducted on a Hitrap Q column (Amersham-Pharmacia) followed by a Superdex 75 S prep-grade column, which had been previously equilibrated with buffer A [50 mM Tris-HCl (pH 7.5), 5% (v/v) glycerol, 200 mM NaCl, 5 mM DTT, and 1 mM TCEP]. The homogeneity of the purified protein was assessed *via* SDS-PAGE (Fig. 1). The protein solution was concentrated using a Centri-Prep (Millipore) to a final concentration of 20 mg/ml with buffer A. Initial crystallization was conducted at 295 K *via* the sitting-drop method, using a Hydra II Plus One (Matrix Technology) with approximately 1,400 conditions, with a ratio of 200 nl of precipitants to 200 nl of protein solution at a temperature of 22°C. Crystallization trials were conducted using screening kits from Hampton Research, Emerald BioStructures, and Jena BioSciences. The crystallization conditions were optimized *via* the hanging-drop vapor-diffusion method using 24-

well plates and a solution of 0.1 M HEPES, pH 8.0, 22% (w/v) PEG 8000, 0.3 M Ca-acetate, and cyclohexyl-methyl-β-D-maltoside as an additive by one-tenth of the total volume used and, later, 1 μl of protein solution mixed with 1 μl of reservoir solution. Each hanging drop was positioned over 1 ml of reservoir solution. The well was sealed with the coverslip using vacuum grease, with the exception of a small leak left between the coverslip and the well. A cryoprotectant solution was developed, which consisted of 0.1 M HEPES, pH 8.0, 22% (w/v) PEG 8000, 0.3 M Ca-acetate, 30% (v/v) glycerol, and cyclohexyl-methyl-β-D-maltoside as an additive with one-tenth of the total volume. The solution was then incubated overnight at 4°C to complete the reaction. The native crystals were soaked in 5 μl of this cryoprotectant solution for 10 s prior to flash-cooling in liquid nitrogen. They were then mounted on the goniometer in a stream of cold nitrogen at 100 K. X-ray diffraction data were collected from the cooled crystals using an ADSC Quantum CCD 210 detector at beamline 4A MXW at Pohang Light Source (PLS), South Korea. The crystal was oscillated by 1.0° per frame over a total range of 200° at a wavelength of 1.0000 Å. X-ray diffraction data to 2.5 Å was collected. Data were integrated and scaled *via* Denzo and Scalepack crystallographic data-reduction routines [6].

EFAlaR was overexpressed in *E. coli* in soluble form with a yield of ~10 mg of homogeneous protein from a liter of culture. The molecular mass of this protein was determined to be 40.1 kDa, according to the results of Superdex 75S gel filtration (Fig. 1). Initially, very irregular crystals were observed. However, after optimization, stick-like crystals were observed with a reservoir solution containing 0.1 M HEPES, pH 8.0, 22% (w/v) PEG 8000, 0.3 M Ca-acetate, and cyclohexyl-methyl-β-D-maltoside, over a period of three days (Fig. 2). Flash-cooled alanine racemase crystals were diffracted to 2.5 Å using 30% (v/v) glycerol as a cryoprotectant. Initially, the diffraction pattern was poor, but this problem was overcome *via* 30 s of annealing in cryo solution. Auto-indexing was conducted with *Denzo*, and the results indicated that the crystals were members of the orthorhombic space group $C222_1$ on the basis of systematic absences, with the following unit cell parameters: a=94.634, b=156.516, c=147.878 Å, and α=β=γ=90°. Two or three monomers are likely present in the asymmetric unit, with corresponding calculated Matthews coefficients, V_M, of 3.38 $Å^3$ Da^{-1} and 2.26 $Å^3$ Da^{-1}, and a solvent content of 63.7% and 45.5%, respectively [3]. Data-collection statistics are provided in Table 1. The structure determination of EFAlaR is currently under way, and the structural details will be described in a separate paper. Our structural information regarding EFAlaR may prove useful in the design and development of new antibiotics.

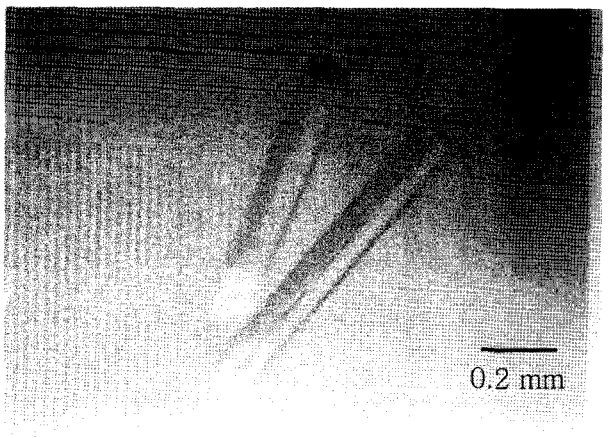

Fig. 2. Crystals of alanine racemase from *Enterococcus faecalis*, used for X-ray diffraction data collection. The crystal size was about 1.2 mm×0.2 mm×0.15 mm.

Table 1. Data-collection statistics (numbers in parentheses are for the outer shell).

X-ray source	Synchrotron (PAL-4A)
Space group	$C222_1$
Resolution (Å)	50–2.5 (2.50–2.59)
No. unique observations	37,447 (3,584)
Completeness (%)	97.9 (95.3)
R_{sym} [a]	0.145 (0.331)
I/σ (I)	17.7 (3.02)

[a] $R_{merge}=\Sigma_h\Sigma_i |I(h)_i-\langle I(h)\rangle| / \Sigma_h\Sigma_i I(h)_i$, where I(h) is the intensity of reflection h, Σ_h is the sum over all reflections, and Σ_i is the sum over i measurements of reflection h.

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