

Communication

Identification and Crystallization of Penicillin-Binding Protein/ β -Lactamase Homolog (Rp46) from *Ruegeria Pomeroyi*

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Abstract: In spite of the enormous biological and clinical significance of penicillin-binding protein (PBP)/ β -lactamase (β L), few of their many homologs (PBP)/ β Ls homologs) have been studied crystallographically, and have known functions. Herein, X-ray crystallographic study of a PBP/ β L homolog (Rp46) from *Ruegeria pomeroyi* is described. Multiple sequence alignments indicate that Rp46 has a conserved serine residue within the S⁷⁰-X-X-K⁷³ motif (Motif I), acting as the catalytic nucleophile. Moreover, an invariant tyrosine residue (Tyr¹⁸⁵) and a Trp³⁶⁵-X-Gly motif (Motif III) were also identified. The recombinant Rp46 protein was expressed in *Escherichia coli* and purified to homogeneity judging from the SDS-PAGE analysis. Rp46 was crystallized using a solution consisting of 20% (*w/v*) PEG 3000, 0.1 M Tris-HCl, pH 7.0, 0.2 M calcium acetate, and the X-ray diffraction data were collected to a resolution of 1.90 Å with an R_{merge} of 7.4%. The crystals of Rp46 belong to the space group *I422*, with unit cell parameters $a = b = 141.26$ Å, and $c = 119.75$. The structure determination and biochemical characterization are in progress. (Synopsis: A penicillin-binding protein/ β -lactamase homolog (Rp46) from *Ruegeria pomeroyi* was identified and crystallized in the space group *I4*, and the diffraction data were collected to a resolution of 1.90 Å.)

Keywords: penicillin-binding protein; lactamase; protein crystal

1. Introduction

Bacterial penicillin-binding proteins (PBPs) and β -lactamases (β Ls) form a large family of serine proteases [1,2]. These enzymes are characterized by phylogenetic analysis, primary sequences, functional properties, and structural similarities. Interestingly, this PBP/ β L family has the nucleophilic serine located within a conserved S-X-X-K motif in the N-terminal region, although most bacterial hydrolases have a highly conserved catalytic motif of G-X-S-X-G in α/β -hydrolase fold [3,4]. To date, the bacterial PBP/ β L family is composed of three classes including low-molecular-weight penicillin-binding proteins (class A to C), high-molecular-weight penicillin-binding proteins (class A to C), and β -lactamases (class A to D) [5–7]. Interestingly, several bacterial enzymes with similar active sites to PBP/ β L proteins, namely PBP- β L homologs, were identified from bacterial organisms including *Klebsiella pneumoniae* [8], *Burkholderia gladioli* [9], *Arthrobacter nitroguajacolicus* [10], *Staphylococcus aureus* [11], *Marinobacter lipolyticus* [12], *Caulobacter crescentus* [13], and *Pseudomonas fluorescens* [14]. Furthermore, these PBP- β L homologs have also been identified from metagenomic

libraries [15–18]. Although physiological roles of these PBP- β L homolog proteins are largely unknown, some of them seem to be involved in the hydrolysis of diverse substrates including DD-peptides [19].

To date, a very limited number of crystal structures of PBP- β L homologs is known including an esterase (EstB) from *Burkholderia gladioli* [20], a simvastatin synthase (LovD) from *Aspergillus terreus* [21], a family VIII carboxylesterase (EstU1) [22], Est-Y29 from a metagenomic library [23], and a PBP-like esterase (CcEstA) [24]. Furthermore, the catalytic mechanisms and physiological functions of this enzyme family remain mainly unclear. In this study, we report the characterization, crystallization, and preliminary crystallographic analysis of a penicillin-binding protein/ β -lactamase homolog (Rp46) from *Ruegeria pomeroyi*. *Ruegeria pomeroyi* is a heterotrophic marine organism essential for understanding the physiology and ecology of *Roseobacter* clade, which consists of ~20% of bacteria in coastal and ocean waters [25]. The crystal structure of Rp46 will provide molecular understanding on the catalytic mechanism and structural features of PBP/ β L homologs.

2. Experimental Procedures

2.1. Cloning, Expression, and Purification of Rp46

The gene coding for Rp46 (GenBank I.D.: AAV95236) was amplified by Polymerase chain reaction (PCR) from the chromosomal DNA of *Ruegeria pomeroyi* (Microbank of Microbial Genomics and Application Center, Daejeon, South Korea). The following primers were used: forward (5'-CAGGATCC ATGCCCCGACCCCGTTAC-3'; BamHI) and reverse (5'-CCAAGCTTCTAAGGAGTCCAAGCTCA-3'; HindIII). This amplification yielded a ~1.2 kb product, including the full length of Rp46 gene. The PCR product was inserted into the pQE30 vector (Qiagen, Hilden, Germany), and the recombinant plasmid (pQE30-Rp46) was used to express the recombinant protein with an N-terminal His-tag (MRGSHHHHHHGS) in *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA, USA). After DNA sequencing, transformed *E. coli* were grown in an LB medium containing 100 μ g/mL of ampicillin at 310 K and 0.1 mM isopropyl- β -D-1-thiogalactoside (IPTG) for the overexpression of Rp46 protein when the OD₆₀₀ reached 0.5. The bacterial culture was grown at 310 K for 4.5 h before centrifugation at 5000 rpm for 20 min at 277 K. The cell pellet was then resuspended in a lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 10 mM imidazole), followed by sonication. The cell lysate was centrifuged at 15,000 rpm for 20 min, and the supernatant was filtered through a 0.22 μ m syringe filter. The supernatants were applied to a Ni-NTA affinity column in an AKTA prime plus system (GE healthcare, Piscataway, NJ, USA), which was previously equilibrated with lysis buffer. After an extensive washing step with lysis buffer containing 40 mM imidazole, the bound Rp46 protein was eluted with elution buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 250 mM imidazole). The fractions of the purified Rp46 were desalted on a PD-10 column (GE healthcare, Piscataway, NJ, USA) using phosphate-buffered saline (PBS) pH 7.4. The purity of Rp46 was confirmed by SDS-PAGE and the concentration was determined using a Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The purified protein was concentrated to 5 mg/mL using Vivaspin concentrators (Vivascience, Westford, MA, USA). The final sample was stored at 253 K without further modification.

2.2. Biochemical Characterization

To identify the hydrolytic activity of Rp46, overlay assay using 4-methylumbelliferyl acetate (4-MU acetate) was performed [26,27]. At first, native-PAGE was performed, followed by staining with Coomassie Brilliant Blue (R-250) to confirm the position of the intact Rp46. Next, the gel was washed with double distilled water for 15 min and next immersed several times in 50 mM sodium phosphate buffer, pH 7.0. Afterwards, 250 μ M 4-MU acetate was added as the substrate. The activity of Rp46 was estimated under UV illumination by measuring the fluorescence generated by the enzyme product 4-methylumbelliferone.

2.3. Crystallization

Crystallization trials of Rp46 were performed by the microbatch method [28] under Al's oil using commercial screening kits Wizard I and II crystallization screening solutions (Emerald BioSystems, Bainbridge Island, WA, USA) at 298 K. A drop (1 μ L screening solution and 1 μ L protein solution) was placed into each well of a Nunc 96-well Mini Tray (Nalge Nunc International, New York, NY, USA). A single crystal grown from the condition of Wizard II No. 18 [20% (*w/v*) PEG 3000, 0.1 M Tris-HCl, pH 7.0, 0.2 M calcium acetate] was used for X-ray diffraction analysis.

2.4. X-ray Data Collection and Data Processing

The Rp46 crystals were then transferred to a cryosolution consisting of the screening solution supplemented with 25% glycerol and flash-frozen in a cold nitrogen gas stream at 100 K prior to data collection. High quality diffraction data were collected using an ADSC Quantum 315 CCD detector (Beamline PAL 4A, Pohang Accelerator Laboratory, Pohang, Korea) at 100 K. The wavelength of the synchrotron X-rays was 1.000 Å and the crystal-to-detector distance was 220 mm. The crystal was rotated through a total of 180° with 0.5° oscillation range at an exposure time of 3 s per frame. The data were processed using the HKL2000 package [29]. The data collection and processing statistics are listed in the Table 1.

Table 1. X-ray data collection and processing statistics values in parentheses are for the highest resolution shell.

Space Group	I422
Unit cell parameters (Å)	$a = b = 141.26, c = 119.75$
Wavelength (Å)	1.000
Resolution (Å)	50.00–1.90 (1.93–1.90)
Unique reflections	92,068 (4589)
Completeness (%)	99.9 (100)
Redundancy	7.6 (7.2)
R_{merge}^1 (%)	7.4 (34.5)
Mean I/σ (I)	44.1 (7.0)

$$^1 R_{\text{merge}} = \sum_{\text{hkl}} |I - \langle I \rangle| / \sum_{\text{hkl}} I.$$

3. Results and Discussion

Rp46 consists of a single 411-amino-acid polypeptide chain with a pI of 5.63. Sequence similarities to Rp46 were annotated mostly as β -lactamases or penicillin-binding proteins (PBPs) in the Conserved Domain Search database (CD search) [30]. Multiple sequence alignments of Rp46 with four sequentially-related hydrolases in the Protein Data Bank (PDB) database [31] indicate that they share the sequence motifs essential for their functionality with low sequence identities (Figure 1). Specifically, Rp46 showed significant sequence identity with EstB from *Burkholderia gladioli* (1CI8, 29.4%), EstU1 from a metagenomic library (4IVI, 32.5%), and Est-Y29 from a metagenomic library (4P6B, 30.1%) (Figure 1). Three highly conserved motifs (Motifs I, II, and III) including the S-X-X-K motif have been previously identified in low-molecular-weight class B penicillin-binding proteins or class C β -lactamases (Figure 2). Rp46 also presents these conserved motifs, indicating that Rp46 shares common structural features with these proteins. In these proteins, a Ser residue (Ser⁷⁰ in Rp46) and a Lys residue (Lys⁷³ in Rp46) from the S-X-X-K motif act as a nucleophile and a general base, and both of them are involved in the formation of oxyanion holes at the active site [32]. The hydroxyl group of tyrosine (Tyr¹⁸⁵ in Rp46) in Motif II is essential for acylation steps [3,33].

Interestingly, there are sequence variations in Motif III among these proteins. In class C β -lactamases, the triad Lys-Thr-Gly is found in the center of Motif III, whereas His-Xaa-Gly is observed in low Molecular weight (MW) class B PBP's [2,3,34]. Therefore, Motif III, unlike the other two motifs, is not highly conserved between low MW class B PBP's and class C β -lactamases. Moreover, Rp46, in common with EstB, EstU1, LovD, and Est-Y29, presents a Trp residue (Trp³⁶⁵) in Motif III, whereas this position is occupied by a His residue in low MW class B PBP's or a Lys residue in class C β -lactamases (Figure 2). Collectively, only a glycine (Gly³⁶⁷ in Rp46) in Motif III among these proteins is strictly conserved. It is interesting to speculate that the sequence differences of Motif III among these proteins could be related to biological functions of this protein family.

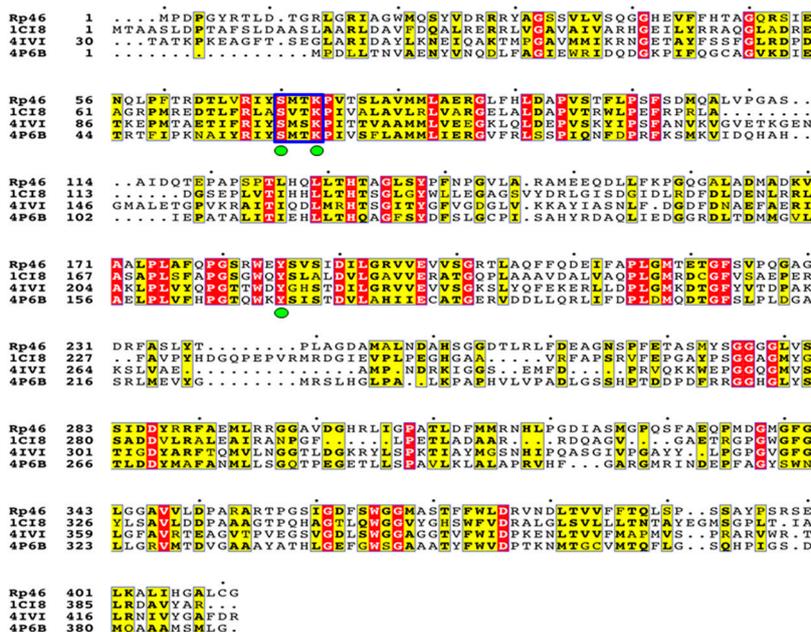


Figure 1. Multiple sequence alignment including Rp46 and three related hydrolases found in the PDB database [31] (1C18: EstB from *B. gladioli* [20]; 4IWI: Family VIII carboxylesterase from uncultured bacterium [22]; 4P6B: PBP- β L homolog from uncultured bacterium [23]). Sequences retrieved from the NCBI server were aligned with CLUSTAL O [35], and the output was rendered using ESPrInt [36]. Identical and highly conserved residues are shown in red and yellow boxes, respectively. The three catalytic residues (Ser, Lys, and Tyr) are labeled with a green circle.

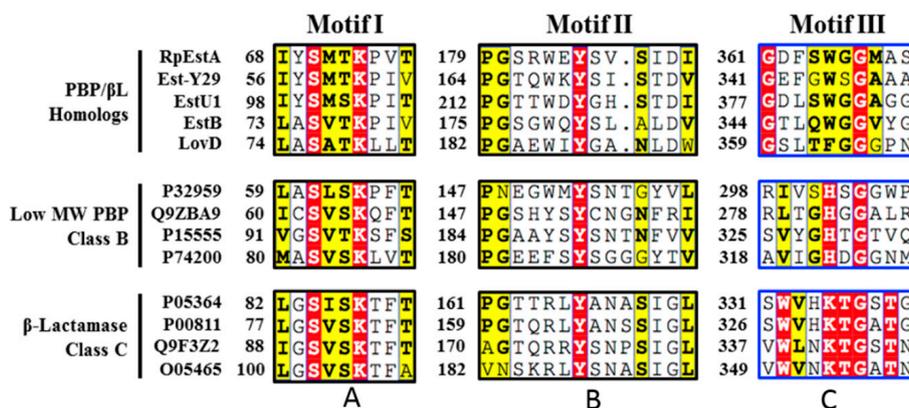


Figure 2. Motifs I, II, and III belonging to PBP- β L homologs, low-molecular-weight class B PBP's, and class C β -Lactamase. Completely conserved residues are shown in red and highly conserved residues are shown in yellow. Note that the catalytic residues of Motifs I and II are highly conserved compared to those of Motif III.

The recombinant Rp46 with an N-terminal His-tag was expressed in *Escherichia coli* and purified to electrophoretic homogeneity for crystallization (Figure 2A). As shown in Figure 3A, the molecular mass of the Rp46 was estimated to be 46 kDa (Figure 2C), which was consistent with the calculated molecular mass (45.98 kDa). The biochemical activity of Rp46 was confirmed on a native Polyacrylamide Gel Electrophoresis (PAGE) gel by zymogram analysis in the presence of 4-MU acetate (Figure 2B).

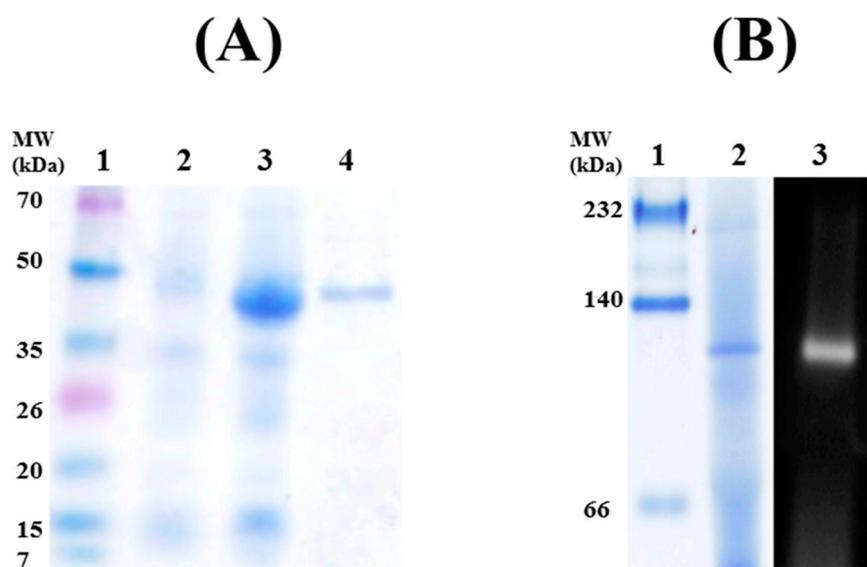


Figure 3. SDS-PAGE and zymography of Rp46. **(A)** SDS-PAGE analysis of protein samples during purification. From left to right, molecular weight markers (1), *E. coli* crude extracts before (2) and after IPTG induction (3), and purified Rp46 (4). **(B)** Zymographic analysis of Rp46. Molecular weight markers (1), Coomassie Brilliant Blue staining after Native-PAGE (2), and overlay activity assay with 4-MU acetate after Native-PAGE (3).

A diffraction-quality crystal of tetragonal shape was obtained under Wizard II-18 condition [20% (*w/v*) PEG 3000, 0.1 M Tris-HCl, pH 7.0, 0.2 M calcium acetate] within two weeks, and grew to final dimensions of $0.3 \times 0.3 \times 0.2 \text{ mm}^3$ (Figure 4). The crystals belonged to the space group *I4* with unit cell parameters $a = b = 141.26 \text{ \AA}$, and $c = 119.75 \text{ \AA}$. The diffraction data set was processed to 1.90 \AA resolution with 99.9% completeness, and R_{merge} value of 7.4%. The data collection statistics are listed in Table 1. Assuming the presence of one molecule per asymmetric unit, Matthews coefficient (V_M) is calculated to be $3.12 \text{ \AA}^3/\text{Da}$. This V_M value is within the range commonly observed for protein crystals, and corresponds to 60.6% solvent content [37]. The structure of Rp46 was searched by molecular replacement using Phenix Phaser-MR [38]. The crystal structure of recently-solved Est-Y29 (PDB ID 4P6B), which has 30.1% sequence identity to Rp46, was used as the search model. The best solution generated by Phaser-MR has $\text{LLG} = 190.0$. Early refinement of this solution by Phenix gives $R_{\text{work}}/R_{\text{free}} = 22.5\%/24.9\%$, indicating the solution is correct. However, manual model building and further refinement are still needed for structural analyses. The biochemical characterization of Rp46 is under progress, and structural and functional studies will elucidate the substrate specificity and the catalytic mechanism of Rp46 in the near future.

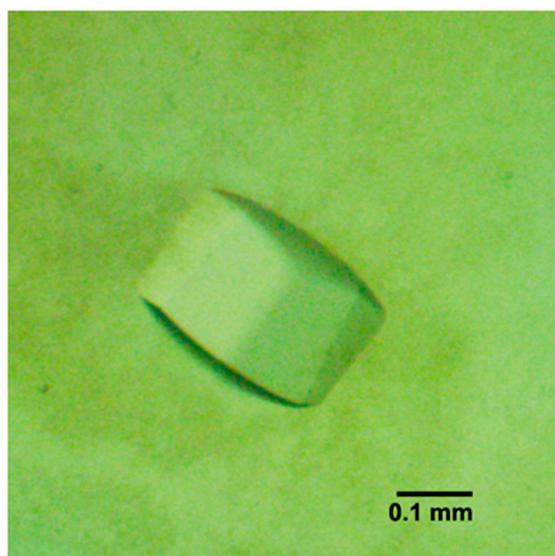


Figure 4. Rp46 crystal was obtained using Wizard II condition No.18 [20% (*w/v*) PEG 3000, 100 mM Tris-HCl, pH 7.0, 200 mM calcium acetate]. The crystal dimensions were $0.3 \times 0.3 \times 0.2$ mm. The scale bar represents 0.1 mm.

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Author Contributions: Bum Han Ryu and T. Doohun Kim identified the gene from the chromosome of *Ruegeria pomeroyi*. Bum Han Ryu, Tri Duc Ngo, and Kyeong Kyu Kim overexpressed and crystallized the protein. The protein was biochemically characterized by Bum Han Ryu, Wanki Yoo, and Tri Duc Ngo. T. Doohun Kim and Kyeong Kyu Kim coordinated the project and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no competing financial interests.

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