Preparation, crystallization and preliminary X-ray crystallographic analysis of OXA-23, a carbapenemase conferring widespread antibiotic resistance

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OXA-23, a class D carbapenemase that confers widespread antibiotic resistance hydrolyzes the β-lactam rings of β-lactam antibiotics, presenting an enormous challenge to infection control, particularly in the eradication of pathogenic bacteria such as Acinetobacter baumannii, one of six top-priority dangerous pathogens. Thus, the enzyme is a potential target for developing antimicrobial agents against pathogens producing carbapenemases. In this study, OXA-23 was purified and crystallized at 298 K and X-ray diffraction data from OXA-23 crystal were collected at 2.03 Å resolution using synchrotron radiation. The crystal of OXA-23 belonged to space group P41 with unit cell parameters a = 82.47, b = 82.47 and c = 172.01 Å. Analysis of the packing density showed that the asymmetric unit probably contained two molecules with a solvent content of 73.64%.

Keywords: Antibiotic resistance, Imipenem, Crystal, OXA-23, Carbapenemase, Acinetobacter baumannii

Serious clinical problems arise from the appearance of antibiotic resistance in pathogenic bacteria causing nosocomial and/or community-acquired infections1-3. β-Lactamas (hydrolyze the β-lactam rings of β-lactam antibiotics and thus provide pathogenic bacteria with resistance to these antibiotics4. Based on their primary structures, β-lactamases have been grouped into four classes (A-D), with classes A, C and D using a serine-based covalent catalysis mechanism5. Class D β-lactamases possess a very high degree of sequence diversity (more than 200 variants) and are particularly troublesome in the eradication of Gram-negative pathogens, such as Acinetobacter baumannii and Pseudomonas aeruginosa4. They have been designated as OXA-type β-lactamases (oxacillinases) because they usually hydrolyze oxacillin (Fig. 1A) better than other penicillins, such as benzylpenicillin (Fig. 1B)6. To overcome the inactivation by these β-lactamases produced mainly by A. baumannii, carbapenems such as imipenem (Fig. 1D) have been developed7.

Imipenem and benzylpenicillin have the very similar core structure, but the size of their R1 and R2 side chains is different. At C6, imipenem has a hydrogen at β position and a hydroxyethyl group at α position, while benzylpenicillin has a phenylacetylamino group at β position and a hydrogen at α position (Fig. 1)8. After clinical use, however, novel OXA-type β-lactamases that can hydrolyze even carbapenems have appeared4. A. baumannii is one of six top-priority dangerous pathogens identified by the Infectious Diseases Society of America and there are few or no drugs in late-stage development for these pathogens, further limiting the choice of an appropriate and safe treatment for these infections2,9.

To date, four main groups of OXA-type carbapenemases, namely OXA-23-like, OXA-24-like, OXA-51-like and OXA-58-like, have been identified in A. baumannii7. OXA-23-like group has been reported worldwide and can hydrolyze oxacillin, cephalothin (Fig. 1C) and imipenem10. Increasing numbers of OXA-type β-lactamases are being found in the clinic, the genes of which have been primarily

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Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; MAD, multi-wavelength anomalous diffraction; OXA, oxacillinase; PCR, polymerase chain reaction; PEG, polyethylene glycol.
located on plasmid or integrons\textsuperscript{11}. This potential for wide dispersal together with the broad substrate specificity and lack of clinically useful inhibitors underlines the importance of investigating the structural (or molecular) details of these enzymes\textsuperscript{11}.

Although various crystal structures for OXA-type $\beta$-lactamases are now available including those for OXA-1, -2, -10, -13, -24, -46, and -48\textsuperscript{12}, but no structures of widespread OXA-23-like $\beta$-lactamases have been thus far reported. Thus, there is a need to determine the crystal structure of OXA-23 for understanding the biochemical characteristics of OXA-23-like carbapenemases and to elucidate structural basis for the expanded catalytic activity of OXA-23 towards carbapenems. Here, we report the cloning, overproduction, crystallization and preliminary X-ray crystallographic analysis of OXA-23 as a first step towards its structure determination.

**Materials and Methods**

**Cloning**

The $\text{bla}_{\text{OXA-23}}$ gene encoding OXA-23 ESBL produced by the *Acinetobacter baumannii* K0420859 was isolated from a urine specimen of a patient hospitalized at a general ward in a tertiary-care Hospital in Busan, Republic of Korea\textsuperscript{13} and amplified by polymerase chain reaction (PCR) using the genomic DNA of the clinical isolate. The sequences of the forward and reverse oligonucleotide primers designed from the published sequence\textsuperscript{13,14} were as follows: 5'-CA TAG TAG ACC ATG GCC CAC CAT CAT CAT GAC GAC GAC GAC AAG ACG GTT CAG CAT AAT TTA ATA-3' (*NcoI* restriction site in bold) and 5'-CAT TGT ACT CTC GAG TTA AAT ATT CAG CTG TTT TAA T-3' (*XhoI* restriction site in bold), respectively; the underlined bases represent a hexa-histidine tag site and the italic bases describe an enterokinase recognition site. The amplified gene was double-digested with *NcoI* and *XhoI* and then inserted into the expression vector pET-28a (Novagen, Madison, WI, USA) that was digested with the same DNA restriction enzymes to produce the pET-28a/His\textsubscript{6}-bla\textsubscript{OXA-23} plasmid. After verifying the DNA sequence, the plasmid DNA was transformed into *E. coli* strain BL21 (DE3) for overexpression of His\textsubscript{6}-OXA-23.

**Overexpression and purification**

The transformed cells were grown in Luria-Bertani medium (Difco, Detroit, MI, USA) containing 50 $\mu$g/mL kanamycin to an OD\textsubscript{600} of 0.5 at 310 K. The expression of His\textsubscript{6}-OXA-23 was induced with 0.5 mM isopropyl-1-thio-$\beta$-D-galactopyranoside (IPTG) for 24 h at 289 K. Cells were harvested by centrifugation at 5000 g (Hanil, Seoul, Korea) for 10 min at 277 K. The resulting cell pellet was resuspended with ice-cold 10 mM Tris-HCl buffer (pH 7.7) and homogenized with sonicator (Sibata, Saitama, Japan). The crude lysate was centrifuged at 20000 g (Haniil) for 30 min at 277 K and the clarified supernatant was loaded on to a His-Bind column (Novagen, Wisconsin, WI, USA) equilibrated with binding buffer (20 mM sodium phosphate, 10 mM imidazole, and 500 mM NaCl pH 7.7). His\textsubscript{6}-OXA-23 was eluted with the same buffer containing 250 mM imidazole. Eluted fractions of His\textsubscript{6}-OXA-23 were pooled and concentrated to a volume of approximately 1 mL by using a VIVA 20 (Sartorius, Goettingen, Germany). For further purification, the His\textsubscript{6} tag was removed from His\textsubscript{6}-OXA-23 by enterokinase according to the manufacturer’s instructions (Roche Molecular Biochemicals, Mannheim, Germany). The reaction mixture was desalted and concentrated by Fast Desalting column (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) and then loaded on to a Superdex 200 prep-grade column (GE Healthcare), which was previously equilibrated with 10 mM Tris-HCl buffer (pH 7.7) for further purification by gel filtration.
The homogeneity of purified protein was analyzed via SDS-PAGE (Fig. 2A). The purified OXA-23 without the His$_6$ tag was dialyzed against 10 mM Tris-HCl buffer (pH 7.7) and subsequently concentrated to 10 mg/mL for the crystallization trials.

**Crystallization**

Crystals of OXA-23 were obtained by the batch-crystallization method at 298 K set up by an automatic crystallization machine (IMPAX 1-5 system; Douglas Instruments Ltd, UK). Small drops composed of 1 µL protein solution and an equal volume of crystallization reagent were pipetted under a layer of a 1:1 mixture of silicon oil and paraffin oil in 72-well HLA plates (Nunc, Roskilde, Denmark). The unique oil formulation allows vapour diffusion from the drop through the oil layer and thus this kind of batch-crystallization method mimics the conventional vapour-diffusion method. Initial crystallization conditions were tested by using all the available screening kits from Hampton Research and Emerald BioStructures Inc (Bainbridge Island, WA, USA). Initial crystal of OXA-23 was grown using a precipitant containing 2.4 M sodium malonate (pH 7.0). The crystallization condition was then optimized to 0.1 M Bis-Tris propane (pH 7.0) and 2.4 M sodium malonate (pH 7.0), which produced larger single crystals suitable for X-ray data collection (Fig. 2B).

**X-ray data collection and processing**

Crystals were cryoprotected with the reservoir solution supplemented with 15% polyethylene glycol (PEG)-400 and flash-frozen in liquid nitrogen. The frozen crystals were then mounted on the goniometer in a stream of cold nitrogen at 100 K. X-ray diffraction data were collected at 2.03 Å using an ADSC Quantum 210 CCD detector of the beamline NW12A at the photon factory (KEK) in Japan (Table 1). The wavelength of the synchrotron radiation was 1.0000 Å. Diffraction data were integrated and scaled via DENZO and SCALEPACK crystallographic data-reduction routines.

**Results and Discussion**

Recombinant OXA-23 from *A. baumannii* was overexpressed in *E. coli* in soluble form (Fig. 2A). The crystal of purified OXA-23 grew to approximate 61.5 × 68.5 × 97.3 µm (Fig. 2B) and belonged to $P4_1$ space group with unit cell parameters $a = 82.47$, $b = 82.47$ and $c = 172.01$ Å. We calculated that the crystal volume per unit of molecular mass ($V_{M}$) was 4.66 Å$^3$ Da$^{-1}$ with a solvent content of 73.64% by volume when the unit cell was assumed to contain two molecules. This corresponded to two molecules per asymmetric unit. The statistics of data collection are summarized in Table 1.

OXA-type β-lactamases represent a heterogeneous group of active site serine β-lactamases that show an extraordinary panel of functional features and substrate profiles, thus representing relevant models for biochemical and structural studies. OXA-type structures suggest the unique, cation-mediated formation of a homodimer. Kinetic and hydrodynamic data show that the dimer is a relevant species in solution and is the more active form of these enzymes. There are significant differences between

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**Table 1—Crystal information and data collection statistics**

<table>
<thead>
<tr>
<th>Data sets</th>
<th>OXA-23 (native)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>$P4_1$</td>
</tr>
<tr>
<td>Unit-cell parameters</td>
<td>82.47, 82.47, 172.01</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Resolution (Å)</td>
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</tr>
<tr>
<td>Completeness (%)</td>
<td>99.6 (98.3)*</td>
</tr>
<tr>
<td>$R_{sym}$ (%)</td>
<td>7.8 (47.1)*</td>
</tr>
<tr>
<td>$I/σ(I)$</td>
<td>9.8</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>73,395</td>
</tr>
<tr>
<td>Average redundancy</td>
<td>6.3 (4.2)*</td>
</tr>
</tbody>
</table>

*The number in parentheses is for the outer shell.

$R_{sym} = \sum |I_{obs}−I_{avg}| / \sum I_{obs}$
active sites observed in two OXA-type carbapenemases (OXA-24 and OXA-48). Comparison of the molecular details of the active site of OXA-24 with the OXA-48 structure reveals that there is no counterpart in OXA-48 to the bridge across the top of the active site formed by residues Y112 and M223 (OXA-24 numbering).

The sequence identities between OXA-23 and the seven OXA-type $\beta$-lactamases with structural information were on average 29% (Fig. 3). Because of lack of OXA-23-like carbapenemase structures, it was challenging to discover the structural basis for the extended catalytic activity of OXA-23 towards carbapenems such as imipenem. Our efforts to determine the structure by molecular replacement by use of AMoRe17 and CNS18 with available structures of the seven OXA-type $\beta$-lactamases as search models were not successful. This suggested OXA-23 having different conformation(s) in the active site, thus explaining the extended catalytic activity towards imipenem compared with seven OXA-type $\beta$-lactamases. Further studies, such as molecular replacement with other programs (or modified models) and growing of selenium-incorporated crystals for multi-wavelength anomalous diffraction (MAD) are in progress.

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