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Crystal structure of *Pseudomonas aeruginosa* PA2196, a putative TetR family transcriptional repressor

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ABSTRACT

PA2196 of *Pseudomonas aeruginosa* is a putative transcriptional regulator and belongs to the TetR family repressor that is involved in adaptations to environmental changes and bacterial antibiotic resistance. The crystal structure of PA2196 determined to 2.4 Å resolution revealed nine α-helical bundles that can be divided into N-terminal DNA binding domain with an α-helix-turn-α-helix motif and C-terminal ligand binding domain with a hydrophobic ligand binding pocket. The distance between the N-terminal domains of homodimeric PA2196 suggested that our structure is similar to the DNA-bound form of other TetR family proteins. The C-terminal ligand binding pocket is composed mainly of hydrophobic residues and has a volume of about 523 $Å^3$ with two openings. PA2196 binds to the upstream region and can regulate the downstream genes that are chemical modification enzymes. Our crystal structure of PA2196 provides insights about the DNA recognition and ligand binding characteristics.

1. Introduction

Pseudomonas aeruginosa is a opportunistic human pathogen that has become a greater threat to human health due to its intrinsic resistance to antibiotics or disinfectants. *P. aeruginosa* has a large proportion of predicted regulatory motifs for adaptations to diverse environmental conditions [1]. One of the transcriptional regulatory motifs is the TetR family repressors, which act as a sensor to changes of the cellular environment and induce target gene expression by being released from the operator DNA upon ligand binding [2]. The major functions of the genes regulated by the TetR family repressors include drug efflux pump [3], multidrug resistance [4–6], modification and clearance of toxic compound [7], osmotic stress regulation [8], and antibiotic biosynthesis [9,10]. The TetR family repressor and its regulated genes are usually adjacent in the genome, and the repressor binds to the upstream or intergenic regions [2]. The ligand recognized by the repressor is often the target molecule of the regulated genes. For example, the TetR repressor is released from the operator DNA when it binds tetracycline and activates downstream genes that remove tetracycline by an efflux pump [11].

The common structural features of TetR family proteins include 10 α-helical bundles divided into two domains and the formation of a homodimer. The N-terminal helices from α1 to α3 form a DNA binding domain with a helix-turn-helix motif (HTH) and recognize a palindromic DNA sequence. Helix α3 is especially important for the specific binding to the target DNA sequence by interacting with major groove of DNA [5]. The C-terminal domain is composed of helices from α4 to α10 and contains a ligand binding pocket of diverse size and properties [2,3,5]. The repressor is released from the operator DNA by ligand-induced conformational changes leading to the widening of the N-terminal DNA binding domains. The exact mechanisms of conformational changes differ among various repressors [3,4,6].

We have determined the structure of PA2196, which is a 2196th gene located at about 2.4 Mbps in the *P. aeruginosa* genome [1]. PA2196 shares sequence and structural similarities with other TetR family repressors. Our structure revealed a typical HTH-motif in the N-terminal domain for specific DNA recognition and a ligand binding pocket in the C-terminal domain. PA2196 might recognize a hydrophobic ligand and regulate downstream genes involved in the modification of its cognate ligand.

2. Materials and methods

2.1. Cloning and protein preparation

The PA2196 gene was amplified from the *P. aeruginosa* genomic DNA by polymerase chain reaction (PCR). The purified PCR product was cloned into the pET28b vector using NheI and EcoRI enzymes with a N-terminal His_6-tag and thrombin-cut site. The construct

Abbreviations: TetR, Tet repressor; HTH, α-helix-turn-α-helix motif; RMSD, root-mean-square deviation.

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Table 1
Data collection and refinement statistics.

Data collection	
Space group	$P2_1$
Unit cell dimensions	a = 60.4, b = 45.6, c = 66.8 Å, β = 109.7°
Resolution (Å)[a]	19.3–2.40 (2.46–2.40)
Observed reflections	314138
Unique reflections	13358
Completeness (%)	98.3 (94.2)
R_{sym} (%)[b]	0.108 (0.422)
I/σ (I)[c]	45.7 (14.3)
Refinement	
No. of residues	374
R_{cryst} (%)/R_{free} (%)[d]	22.1/29.7
Rmsd bonds (Å)	0.016
Rmsd angles (°)	1.56

[a] Resolution range of the highest shell is listed in parentheses.
[b] $R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where I is the intensity of an individual reflection and $\langle I\rangle$ is the average intensity over symmetry equivalents.
[c] I/σ (I) is the mean reflection intensity/estimated error.
[d] $R_{cryst} = \sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes, R_{free} is equivalent to R_{cryst} but calculated for a randomly chosen set of reflections that were omitted from the refinement process.

was transformed into Rosetta (DE3) *Escherichia coli* strain (Novagen). The cells were grown in LB media containing 30 μg/ml kanamycin at 37 °C until the optical density at 600 nm reached 0.6. Protein expression was induced at 20 °C with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were lysed by sonication in 20 mM Tris–HCl pH 7.5 and 250 mM NaCl buffer (lysis buffer). The lysate was cleared by centrifugation and the supernatant was loaded onto a Ni-Sepharose 6 affinity column and eluted with stepwise gradient of 50–400 mM imidazole in lysis buffer. After the N-terminal His_6-tag from the vector was cut by thrombin at 4 °C, PA2196 was further purified using a Superdex75 size-exclusion column (GE Healthcare) equilibrated with a buffer composed of 20 mM Tris–HCl, 200 mM NaCl, 2 mM dithiothreitol, and 2 mM EDTA. The purity of the protein was analyzed by SDS–PAGE.

2.2. Crystallization, data collection and structure determination

The purified PA2196 was concentrated to 11 mg/ml by centrifugal ultrafiltration (Amicon). Crystals of PA2196 were obtained by a hanging-drop vapor-diffusion method at 20 °C using a well solution composed of 20% PEG 3350, 0.2 M sodium formate, and 13% dimethylsulfoxide (DMSO). Crystals were transferred into 20% PEG 3350, 0.2 M sodium formate, and 20% DMSO as a cryoprotectant solution and flash-frozen in liquid nitrogen. X-ray diffraction data were collected to 2.4 Å resolution at the Photon factory (PF, Japan). The data were processed with HKL2000 [12] and an initial model of PA2196 was obtained by molecular replacement using the MOLREP program in the CCP4 package [13] with the putative TetR/AcrR family transcription regulator of *Acinetobacter* sp. ADP1 (PDB ID: 3KNW) as a search model. The space group is a monoclinic $P2_1$ and the asymmetric unit contains two subunits that form a homodimer (Table 1). The Matthews coefficient (Vm) is 1.99 $Å^3$/Da, and the estimated solvent content of the crystal is 38.2%. The model was refined with REFMAC and manual model building was performed using the COOT program [14]. Six residues (Met1 – Asp6) in the N-terminal region and last residue (Ile194) were not observed in the electron density and were not included in the final model. The Ramachandran plot produced by PROCHECK showed that 100% of residues are in the most favored or favored region [15].

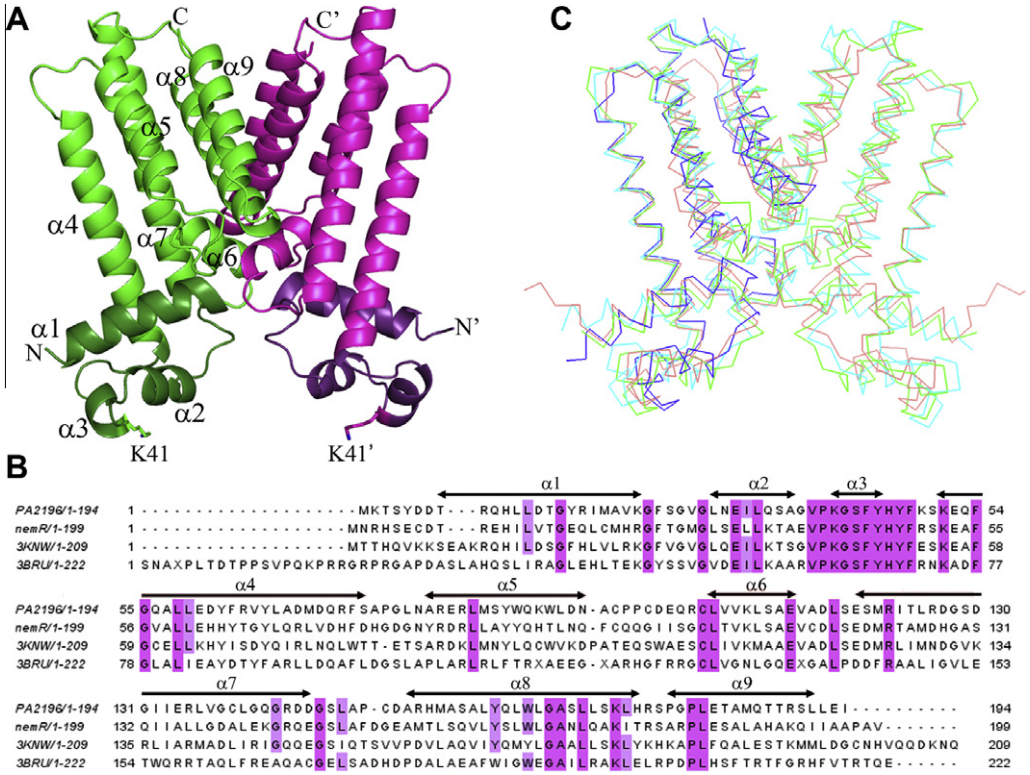

Fig. 1. Structure of PA2196 and comparison with homologs. (A) Overall structure of PA2196 dimer. The N-terminal domain (α1–α3) of subunit A is colored in dark green and the C-terminal domain (α4–α9) in green. The corresponding domains in subunit B are colored in dark magenta and magenta, respectively. The N- and C-termini are labeled as N and C. The K41's in subunits A and B were used to measure the distance between the N-terminal domains and are shown in a ball-and-stick format. (B) Sequence alignment of *Pseudomonas aeruginosa* PA2196, *Escherichia coli* nemR, *Acinetobacter baylyi* 3KNW (PDB ID), *Rhodobacter sphaeroides* 3BRU (PDB ID). The residues are colored by conservation level. The secondary structure of PA2196 is indicated above the sequence. (C) Superimposition of PA2196 (magenta), 3KNW (blue) and 3BRU (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. DNA binding assays

The upstream 153 bp DNA of the PA2196 was prepared by PCR from *P. aeruginosa* genomic DNA using primers (5′-GAATTGTTTC-TAGACGACTGGTCTAATTCAAGCTA-3′ and 5′-TAGCTTGAATTAGACC AGTCGTCTAGAAACAATTC-3′) and the semi-palindromic 25 bp DNA (5′-GTTTCTAGACGACTGGTCTAATTCA-3′) in the upstream region was synthesized. The upstream 153 bp DNA (1 pmol) or 25 bp oligomer (2.5 pmol) was mixed with increasing amount of PA2196 with molar ratios (protein/DNA) of 4, 8, 12 in the binding buffer (20 mM Tris–HCl 8.0, 3 mM magnesium acetate). After incubation at 20 °C for 30 min, the sample was loaded to 12% TBE-polyacrylamide gel and stained with ethidium bromide for visualization. The binding affinity between DNA and PA2196 was calculated using the Prism software (Graphpad) based on the intensity of the DNA bands analyzed by the ImageQuant analysis program (Amersham Bioscience).

2.4. Protein Data Bank accession number

The coordinate and structure factors for *P. aeruginosa* PA2196 have been deposited in the RCSB Protein Data Bank with accession code 3RD3.

3. Results and discussion

3.1. Overall structure of PA2196

The crystal structure of PA2196 was determined to 2.4 Å resolution by molecular replacement. PA2196 forms a homodimer composed mostly of α-helices and the overall structure of PA2196 is very similar to those of other TetR family proteins (Fig. 1A) [2]. Structural comparison with other related proteins identified by DALI [16] showed that PA2196 is closest to 3KNW and 3BRU with RMSD's of 1.22 and 1.78 Å, respectively (Fig. 1C). Each monomer of PA2196 is composed of an N-terminal DNA binding domain (from α1 to α3) and a C-terminal ligand binding domain (from α4 to α9). In the N-terminal domain, the α2 and α3 helices form the canonical HTH motif that can interact with the major groove of the DNA (Fig. 1A). Each C-terminal domain contains a ligand binding pocket, and α-helices α6, α8, and α9 are involved in the dimerization of the two subunits.

3.2. N-terminal DNA binding domain

The N-terminal DNA binding domain contains the typical helix-turn-helix motif. Previous studies of other TetR family proteins showed that conformational changes upon ligand binding led to the widening of N-terminal DNA-binding domains [3]. Center-to-center distance of the N-terminal domains measured between the Cα atoms of two K41's in subunit A and B is about 35 Å (Fig. 1A). The corresponding distances of the other transcriptional regulators, AcrR and QacR, are 35 and 37 Å, respectively, in the DNA-bound form [6,17]. Because the distance between adjacent major grooves of standard B-form DNA is about 34 Å, the HTH motif of PA2196 can potentially fit into the major grooves of DNA for binding. Multiple sequence alignment with related proteins shows a relatively low sequence conservation level except helix α3 (Fig. 1B). Helix α3 contains residues K41, G42 and S43 that were shown to interact with the DNA base-pairs in the major groove. Other well-conserved residues such as Y45, H46, Y47 and K51 are known to interact with the phosphate backbone of DNA [2,17].

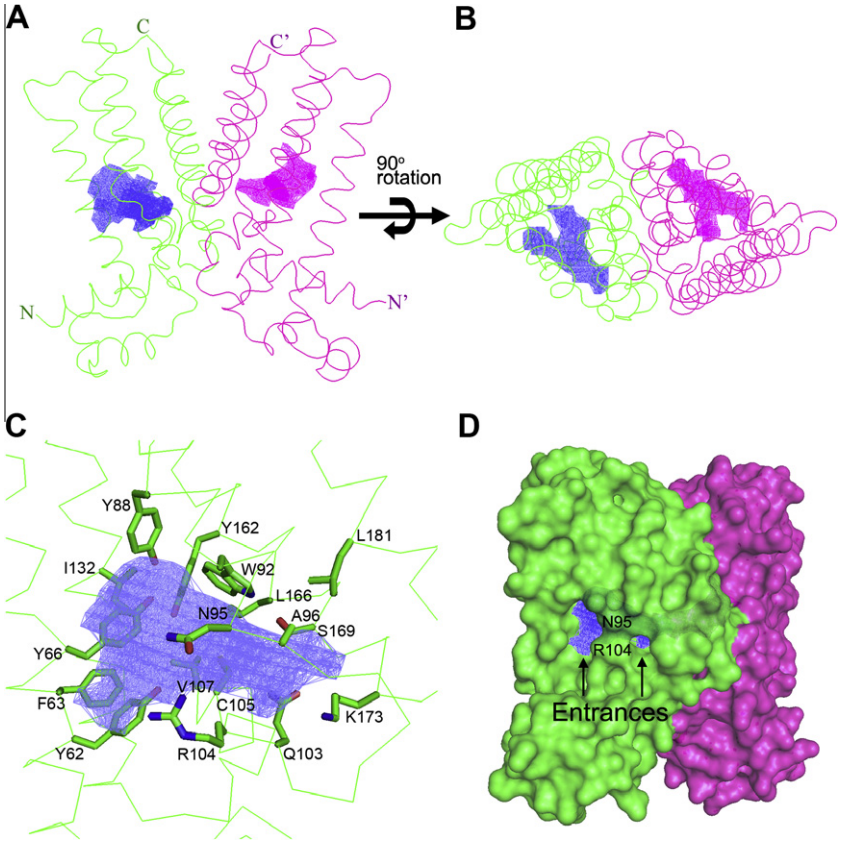

Fig. 2. Ligand-binding pocket of PA2196. (A) The ligand-binding pocket in the C-terminal domain found by the Pocket Finder program is shown in mesh. The pockets in subunit A and B are colored in blue and magenta, respectively. (B) A 90° rotated view of (A). (C) Residues that form the pocket in subunit A are shown in ball-and-stick model. (D) Surface representation of PA2196 shows two entrances into the pocket near residues N95 and R104. The orientations of PA2196 are same in (C and D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. C-terminal ligand binding domain

Each subunit of the PA2196 homodimer contains a ligand binding pocket in the C-terminal domain (Fig. 2A and B). The volume of the pocket is about 523 Å^3 calculated with the Pocket Finder program [18]. The pocket has two entrances close to residues R104 and N95 (Fig. 2D). The pocket is surrounded mainly by hydrophobic residues including Y62, F63, Y66, Y88, W92, N95, A96, Q103, R104, C105, V107, I132, Y162, L166, S169, K173 and L181 (Fig. 2C). These residues forming the pocket are variable even among the close homologs of TetR family proteins, except C105, K173 and L181, suggesting diverse ligand binding properties of the pocket (Fig. 1B). C105 is in the middle of the pocket and K173 forms a positively-charged patch in the corner of the pocket and may prefer the binding of negatively-charged ligand molecules. The identity of the ligand(s) of PA2196 is not yet known. However, the ligand is speculated to be mainly hydrophobic with negative charges based on the property of the residues forming the ligand binding pocket.

The TetR family repressors usually bind to incomplete palindromic sequences in the upstream region of their own gene or an intergenic region between the repressor and regulated genes. Because of the sequence similarity between PA2196 and nemR in helix α3 (Fig. 1B), we hypothesized that PA2196 and nemR may recognize a similar DNA sequence. The nemR binds to an upstream region of its own gene and recognizes a semi-palindromic DNA sequence (TAGACCGACTGGTCTA). We identified a similar sequence in the upstream region of PA2196 gene (TAGACGACTGGTCTA). The DNA between PA2195 and PA2196 (upstream 153 bp DNA) was amplified using PCR and its binding to the PA2196 was examined by an electrophoretic mobility shift assay (EMSA) (Fig 3).

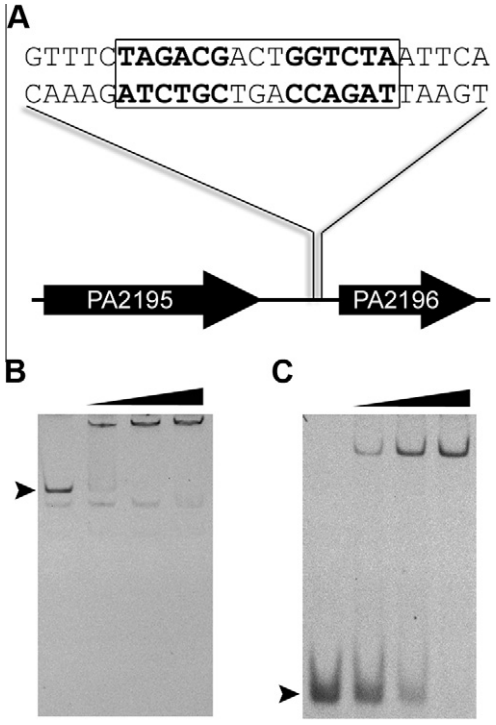

Fig. 3. Binding assay of PA2196 and upstream DNA. (A) The location and sequence of the 25 bp DNA in the upstream region of PA2196 are shown. The putative operator region is boxed and the semi-palindromic sequences are shown in bold. (B) The migration of 153 bp upstream DNA (indicated by arrow) changes with increasing amount of PA2196 but the band below 153 bp DNA (a non-specific PCR product) does not change its migration pattern. (C) Binding assay with 25 bp semi-palindromic DNA (indicated by arrow) in the upstream region.

PA2196 binds to the upstream DNA region with K_d of about 430 nM (Fig 3B). We also used the 25 bp oligomer that contains the semi-palindromic DNA sequence (TAGACG-ACT-GGTCTA) in the upstream region for binding assay with PA2196 (Fig 3C). The 25 bp oligomer showed a comparable affinity (K_d = 2.4 μM) to the 153 bp upstream DNA, suggesting that this semi-palindromic region is the recognition site of PA2196. These results showed that PA2196 can bind to upstream DNA by recognizing a semi-palindromic sequence to regulate the downstream genes.

PA2197, 2198 and 2199 are the downstream genes of PA2196, and their functions are predicted to be a NADP-dependent oxidoreductase, antibiotic biosynthesis monooxygenase and 3-hydroxyisobutyrate dehydrogenase, respectively. These predicted functions suggest that PA2196 may regulate chemical modifying enzymes rather than efflux pump. For example, nemR, a member of the TetR family repressors recognizes cytotoxic *N*-ethylmaleimide (NEM) and regulates nemA gene coding the NEM reductase, whose function is the modification and degradation of NEM [7]. 3KNW and 3BRU, other close homologs of PA2196 found by DALI (Fig. 1B), also have a downstream gene whose function is predicted to be NADP-dependent Zn binding oxidoreductase. Hence, the function of PA2196 is thought to be activating the downstream genes by releasing from the operator region after recognition of toxic ligand molecules.

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References

[1] C.K. Stover, X.Q. Pham, A.L. Erwin, S.D. Mizoguchi, P. Warrener, M.J. Hickey, F.S. Brinkman, W.O. Hufnagle, D.J. Kowalik, M. Lagrou, R.L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L.L. Brody, S.N. Coulter, K.R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G.K. Wong, Z. Wu, I.T. Paulsen, J. Reizer, M.H. Saier, R.E. Hancock, S. Lory, M.V. Olson, Complete genome sequence of *Pseudomonas aeruginosa* PAO1 an opportunistic pathogen, Nature 406 (2000) 959–964.

[2] J.L. Ramos, M. Martinez-Bueno, A.J. Molina-Henares, W. Teran, K. Watanabe, X. Zhang, M.T. Gallegos, R. Brennan, R. Tobes, The TetR family of transcriptional repressors, Microbiol. Mol. Biol. Rev. 69 (2005) 326–356.

[3] P. Orth, F. Cordes, D. Schnappinger, W. Hillen, W. Saenger, W. Hinrichs, Conformational changes of the Tet repressor induced by tetracycline trapping, J. Mol. Biol. 279 (1998) 439–447.

[4] M.A. Schumacher, M.C. Miller, S. Grkovic, M.H. Brown, R.A. Skurray, R.G. Brennan, Structural mechanisms of QacR induction and multidrug recognition, Science 294 (2001) 2158–2163.

[5] M.D. Routh, C.C. Su, Q. Zhang, E.W. Yu, Structures of AcrR and CmeR: insight into the mechanisms of transcriptional repression and multi-drug recognition in the TetR family of regulators, Biochim. Biophys. Acta 1794 (2009) 844–851.

[6] R. Gu, M. Li, C.C. Su, F. Long, M.D. Routh, F. Yang, G. McDermott, E.W. Yu, Conformational change of the AcrR regulator reveals a possible mechanism of induction, Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 64 (2008) 584–588.

[7] Y. Umezawa, T. Shimada, A. Kori, K. Yamada, A. Ishihama, The uncharacterized transcription factor YdhM is the regulator of the nemA gene encoding *N*-ethylmaleimide reductase, J. Bacteriol. 190 (2008) 5890–5897.

[8] T.P. Rkenes, T. Lamark, A.R. Strom, DNA-binding properties of the BetI repressor protein of *Escherichia coli*: the inducer choline stimulates BetI–DNA complex formation, J. Bacteriol. 178 (1996) 1663–1670.

[9] R. Natsume, R. Takeshita, M. Sugiyama, Y. Ohnishi, T. Senda, S. Horinouchi, Crystallization of CprB an autoregulator-receptor protein from *Streptomyces coelicolor* A3(2), Acta Crystallogr. D Biol. Crystallogr. 59 (2003) 2313–2315.

[10] H. Kinoshita, H. Ipposhi, S. Okamoto, H. Nakano, T. Nihira, Y. Yamada, Butyrolactone autoregulator receptor protein (BarA) as a transcriptional regulator in *Streptomyces virginiae*, J. Bacteriol. 179 (1997) 6986–6993.

[11] W. Hinrichs, C. Kisker, M. Duvel, A. Muller, K. Tovar, W. Hillen, W. Saenger, Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance, Science 264 (1994) 418–420.

[12] Z. Otwinowski, W. Minor, Processing of X-ray diffraction data collected in oscillation mode, Methods Enzymol. 276 (1997) 307–326.

[13] A. Vagin, A. Teplyakov, Molecular replacement with MOLREP, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 22–25.

[14] P. Emsley, K. Cowtan, Coot: model-building tools for molecular graphics, Acta Crystallogr. D Biol. Crystallogr. 60 (2004) 2126–2132.
[15] A.L. Morris, M.W. MacArthur, E.G. Hutchinson, J.M. Thornton, Stereochemical quality of protein structure coordinates, Proteins 12 (1992) 345–364.
[16] L. Holm, P. Rosenstrom, Dali server: conservation mapping in 3D, Nucleic Acids Res. 38 (2010) W545–549.
[17] M.A. Schumacher, M.C. Miller, S. Grkovic, M.H. Brown, R.A. Skurray, R.G. Brennan, Structural basis for cooperative DNA binding by two dimers of the multidrug-binding protein QacR, EMBO J. 21 (2002) 1210–1218.
[18] M. Hendlich, F. Rippmann, G. Barnickel, LIGSITE: automatic and efficient detection of potential small molecule-binding sites in proteins, J. Mol. Graph Model 15 (1997) 359–363, 389.