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Patrick Weber,[a] Djalal Meziane-Cherif,[b]* Ahmed Haouz,[a] Frederick A. Saul[c] and Patrice Courvalin[b]

[a]Institut Pasteur, Plateforme 6, CNRS URA 2185, 25 rue du Dr Roux, 75724 Paris Cedex 15, France, [b]Institut Pasteur, Unité des Agents Antibactériens, 75724 Paris Cedex 15, France, and [c]Institut Pasteur, Unité d'Immunologie Structurale, CNRS URA 2185, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

Correspondence e-mail: djalal.meziane-cherif@pasteur.fr

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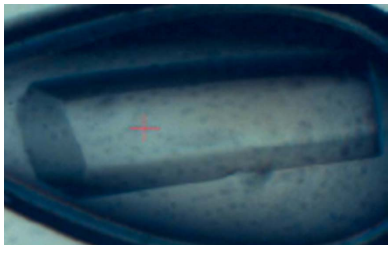

Acquired VanG-type resistance to vancomycin in *Enterococcus faecalis* BM4518 arises from inducible synthesis of peptidoglycan precursors ending in D-alanyl-D-serine, to which vancomycin exhibits low binding affinity. VanG, a D-alanine:D-serine ligase, catalyzes the ATP-dependent synthesis of the D-Ala-D-Ser dipeptide, which is incorporated into the peptidoglycan synthesis of VanG-type vancomycin-resistant strains. Here, the purification, crystallization and preliminary crystallographic analysis of VanG in complex with ADP are reported. The crystal belonged to space group $P3_121$, with unit-cell parameters $a = b = 116.1$, $c = 177.2$ Å, and contained two molecules in the asymmetric unit. A complete data set has been collected to 2.35 Å resolution from a single crystal under cryogenic conditions using synchrotron radiation.

1. Introduction

Peptidoglycan is an essential component of the bacterial cell wall. It contributes to cell integrity and maintenance of a defined cell shape and is intimately involved in cell growth and division. Glycopeptide antibiotics such as vancomycin and teicoplanin target the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of intermediates in peptidoglycan synthesis, preventing the transpeptidation and transglycosylation reactions that lead to a mechanically strong cell wall (Reynolds, 1989). The D-Ala residues that interact with the drug are incorporated as dipeptides that are synthesized in an ATP-dependent fashion by the chromosomal D-Ala:D-Ala ligase. Resistance to vancomycin-type antibiotics arises from the acquisition of ligases of altered specificity that catalyze the formation of an ester bond between D-Ala and D-lactate (D-Lac; VanA/VanB/VanD-type resistance) or a peptide bond between D-Ala and D-serine (D-Ser; VanC/VanE/VanG-type resistance) and to elimination of the high-affinity D-Ala-D-Ala-ending precursors synthesized by the host D-Ala:D-Ala ligase. The modified peptidoglycan precursors exhibit a 1000-fold and sixfold decrease in binding affinity to vancomycin, respectively, accounting for the difference in resistance levels (Arthur *et al.*, 1992; Reynolds *et al.*, 1994).

To study the molecular basis of D,D-ligase specificity, the X-ray structures of *Escherichia coli* DdlB D-Ala:D-Ala ligase and *Enterococcus* VanA D-Ala:D-Lac ligase have been solved at 2.3 and 2.5 Å resolution, respectively (Fan *et al.*, 1994; Roper *et al.*, 2000). Both were in complex with a phosphinophosphate inhibitor that is a close analogue of a tetrahedral intermediate in the normal catalytic reaction, allowing definition of the active-site region. Although the overall topology of the active sites of DdlB and VanA is very similar, important differences were observed, particularly in the hydrophobic region bordered by Leu282 and Tyr216 in DdlB (corresponding to Arg317 and His244 in VanA). The selectivity of VanA for D-Lac could arise from the positive charge of His244, which would attract the negatively charged D-Lac and reject the protonated (NH_3^+) form of D-Ala at the second subsite (Roper *et al.*, 2000). The situation in the D-Ala:D-Ser ligase is still unclear. Although there is obvious general sequence similarity between VanA, DdlB and the D-Ala:D-Ser ligases, important differences occur in the active-site region. Mutational analysis and structural modelling of the VanC2 D-Ala:

D-Ser ligase from *E. casseliflavus* on the basis of the X-ray structure of DdlB predicted that Arg322 and Phe250, which are conserved in all D-Ala:D-Ser ligases, could be responsible for the greater affinity of D-Ser in the second binding site of VanC2 (Healy *et al.*, 1998). The guanidino group of Arg322 probably makes hydrogen bonds to the hydroxyl side chain of D-Ser; however, the role of Phe250 has not been determined.

To date, no crystal structures of D-Ala:D-Ser ligases have been determined. Here, we report the crystallization and preliminary X-ray characterization of VanG complexed with ADP. Understanding the molecular basis of the D-*X* switching among members of the D-Ala:D-*X* ligase family should help in decoding antibiotic resistance and designing effective ligase inhibitors.

2. Materials and methods

2.1. Cloning, overproduction and purification of VanG

Oligodeoxynucleotides EfLBsaF (5′-**GGTCTC**CCATGCAAAA-TAAAAAAATAGCAG-3′) and EfLXhoR (5′-**CTCGAG**TTCCA-CATACAGACCTATCAGC-3′) containing *Bsa*I and *Xho*I restriction sites (bold) were used to amplify the *vanG* gene from *E. faecalis* BM4518 with *Pfu* DNA polymerase. The PCR products were cloned in the pCR-Blunt vector, resequenced and subcloned under the control of the T7 promoter in pET28a(+) previously digested with *Nco*I and *Xho*I enzymes, producing pAT911 [pET28a(+)Ω*vanG*]. The VanG protein, engineered to have a C-terminal His_6 tag, was purified using a protocol derived from that of Lessard *et al.* (1999). Freshly transformed *E. coli* BL21(DE3)-pREP4 harbouring plasmid pAT911 was grown in 1 l LB medium containing 25 µg ml^{-1} kanamycin and 100 µg ml^{-1} ampicillin. The culture was grown at 310 K to an OD_{600} of 0.8, at which point isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 m*M*. After 4 h induction at 301 K, the cells were harvested by centrifugation and resuspended in 8 ml buffer *A* [50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl, 10%(*v*/*v*) glycerol] supplemented with 10%(*v*/*v*) BugBuster 10× Protein Extraction Reagent (Novagen, Madison, Wisconsin, USA), 25 units Benzonase (Sigma–Aldrich) and 2.5 m*M* imidazole. The mixture was stirred for 15 min at room temperature. Cellular debris was removed by centrifugation at 20 000*g* for 45 min and the supernatant was applied onto a 5 ml HisTrap Fast Flow column (GE Healthcare, Uppsala, Sweden) equilibrated with buffer *A* containing 40 m*M* imidazole. The VanG protein was eluted with buffer *A* with a gradient of 40–500 m*M* imidazole over 30 ml. Fractions containing the recombinant His_6-VanG were analyzed by SDS–PAGE. Enzyme activity was determined using a spectrophotometric assay monitored at 340 nm, in which the production of ADP is coupled to the oxidation of NADH by pyruvate kinase and L-lactate dehydrogenase (Daub *et al.*, 1988). The fractions containing VanG were pooled, dialyzed overnight against 3 l 50 m*M* HEPES pH 7.5, 150 m*M* KCl and 1 m*M* EDTA, concentrated to 14.5 mg ml^{-1} with a Centriprep 30 concentrator and stored at 193 K. The molecular mass of the purified VanG was determined by MALDI–TOF mass spectrometry.

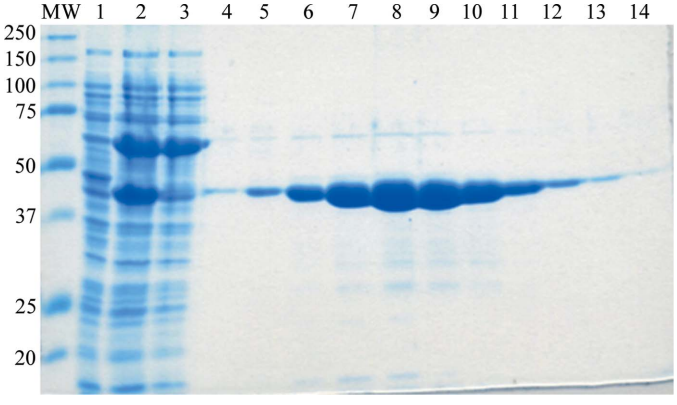

Figure 1
SDS–PAGE analysis of VanG purification. Lane 1, crude extract without IPTG; lane 2, crude extract after 1 m*M* IPTG induction; lane 3, flowthrough; lanes 4–14, eluted VanG fractions.

2.2. Crystallization

Initial crystallization screening was carried out by the vapour-diffusion method using a Cartesian nanolitre dispensing system (Santarsiero *et al.*, 2002). Sitting drops containing 200 nl protein solution at a concentration of 14.5 mg ml^{-1} with 15 m*M* ADP and 200 nl crystallization solution were equilibrated against 150 µl buffer solution in Greiner plates. Screening trials (480 conditions) were performed using commercially available sparse-matrix kits: Structure Screens 1 and 2 from Molecular Dimensions Ltd, JSB Screens 1–8 from Jena Biosciences, Crystal Screen 1 and 2 from Hampton Research and Wizard I and II from Emerald BioSystems. Successful results were obtained using ammonium sulfate and lithium sulfate as precipitant. The crystallization conditions were reproduced and optimized using crystallization solutions generated by a Matrix Maker automated formulation system from Emerald BioSystems.

2.3. Data collection

A single crystal of the VanG–ADP complex was flash-cooled in liquid nitrogen using reservoir solution containing 25%(*v*/*v*) glycerol as cryoprotectant. X-ray diffraction data were collected on beamline PROXIMA 1 at the SOLEIL synchrotron (St Aubin, France). Diffraction images were measured using an ADSC Quantum 315r CCD detector, with a rotation of 0.5° per image at a wavelength of 0.98 Å. The images were integrated with the program *XDS* (Kabsch, 1988) and processed using the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The VanG protein was purified by nickel-affinity chromatography to yield 42 mg protein from 1 l culture. SDS–PAGE analysis showed that the preparation was free of major and minor contaminating species (Fig. 1). The subunit molecular mass of VanG was confirmed as

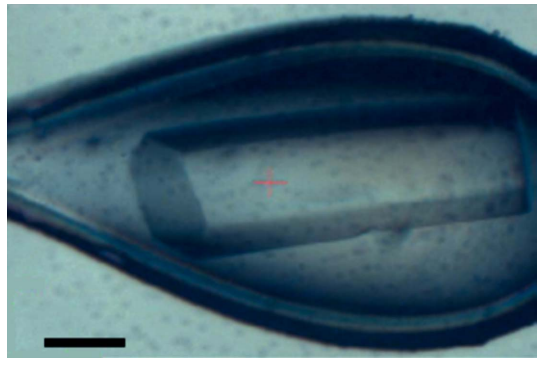

Figure 2
Crystal of VanG D-Ala:D-Ser ligase obtained in the presence of ADP grown in 0.5 *M* ammonium sulfate, 0.9 *M* lithium citrate and 0.1 *M* sodium citrate pH 5.6. The crystals of VanG appeared as hexagonal rods with dimensions of up to 0.06 × 0.06 × 0.2 mm. The scale bar represents 50 µm.

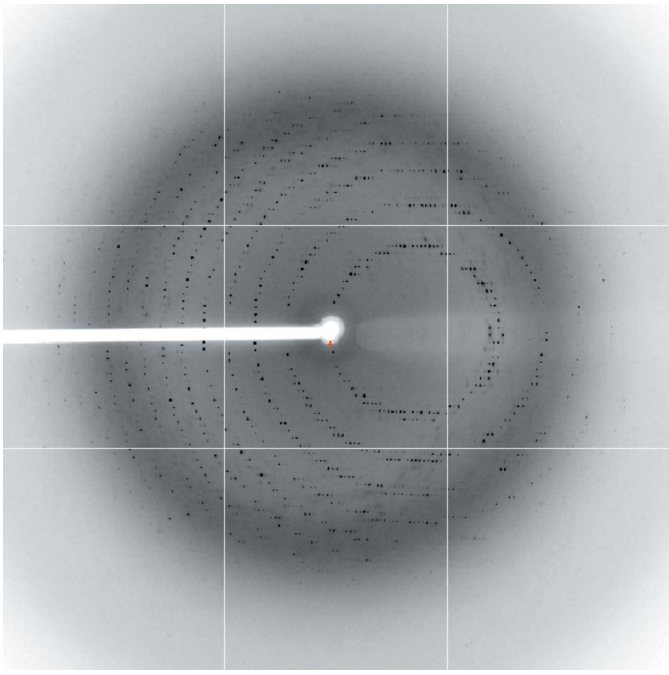

Figure 3
X-ray diffraction image from crystal of VanG in complex with ADP. The image was obtained at Synchrotron SOLEIL. Diffraction extended to a resolution of 2.35 Å.

39 947 Da, compared with a calculated molecular weight of 39 932 Da. This value was determined from the amino-acid sequence of VanG including the LEHHHHHH C-terminal tag sequence. The specific activity of VanG for synthesis of D-Ala-D-Ser was found to be 8.2 µmol min^{-1} mg^{-1}.

The best crystals were obtained at 291 K using the hanging-drop method by mixing 2 µl protein solution containing 10 m*M* ADP with 2 µl crystallization solution containing 0.5 *M* ammonium sulfate, 0.9 *M* lithium sulfate and 0.1 *M* sodium citrate pH 5.6 and equilibrating against 1 ml reservoir solution. Crystals appeared after one week as hexagonal rods with dimensions of up to 0.06 × 0.06 × 0.2 mm (Fig. 2). Initial data processing indicated that the crystal belonged to the trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 116.1$, $c = 177.2$ Å, and diffraction extended to a resolution of 2.35 Å. A representative diffraction image is shown in Fig. 3.

A search for homologous sequences in the Protein Data Bank (Berman *et al.*, 2000) for phasing using the molecular-replacement method was carried out using *FASTA* (Mackey *et al.*, 2002) available from the EMBL–EBI website (http://www.ebi.ac.uk/). Molecular-replacement calculations were carried out with the program *Phaser* (McCoy *et al.*, 2007) as implemented in the *CCP*4 package. The molecular-replacement search model was based on the atomic coordinates of the VanA D-Ala:D-Lac ligase from *E. faecium* BM4147 (PDB code 1e4e; Roper *et al.*, 2000), which has 45% sequence identity with VanG.

Molecular-replacement calculations were performed for both possible space groups $P3_121$ and $P3_221$. A clear solution was obtained in $P3_121$ for two independent molecules forming a dimer in the asymmetric unit. The translational *Z*-score values (TFZ; McCoy *et al.*, 2007) were 10.2 and 14.1, respectively, for molecules 1 and 2 in space group $P3_121$, with increasing log-likelihood gain (LLG; McCoy *et al.*, 2007) values of 57 and 81, respectively, for one-molecule and two-molecule solutions. For space group $P3_221$, the corresponding highest translational *Z*-score value was 5.3 and no positive LLG values were obtained for more than a single molecule. These results allowed us to assign the space group as $P3_121$. No molecular-replacement solution was obtained for more than two molecules of VanG in the asymmetric unit. The solution corresponds to a high solvent content (V_s) of 72% and a Matthews coefficient (V_M) of 4.5 $Å^3$ Da^{-1} (Matthews, 1968; Kantardjieff & Rupp, 2003). Initial calculations of electron-density maps based on the molecular-replacement solution clearly revealed density for the ADP moiety in the binding site of the two independent molecules of VanG in the asymmetric unit (data not shown). A summary of the crystal parameters and data statistics is shown in Table 1.

Table 1
Crystallographic parameters and data statistics.

Values in parentheses are for the high-resolution shell.

Space group	$P3_121$
Unit-cell parameters (Å)	$a = b = 116.1$, $c = 177.2$
Data resolution (Å)	33.5–2.35 (2.48–2.35)
Unique reflections	57000 (8252)
Multiplicity	3.7 (3.8)
Completeness (%)	98.5 (98.6)
R_{merge}†	0.066 (0.778)
$\langle I/\sigma(I)\rangle$	12.3 (1.9)
Molecules per ASU	2
V_M ($Å^3$ Da^{-1})	4.5
Solvent content (%)	72

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

A model of the VanG–ADP complex is currently being built and refined. We are also seeking to crystallize the protein in the apo form or in complex with a transition-state analogue in order to understand the specificity and reaction mechanism of this ligase.

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