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Structural basis for the substrate recognition of peptidoglycan pentapeptides by *Enterococcus faecalis* $VanY_B$

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ABSTRACT

Vancomycin resistance in *Enterococci* and its transfer to methicillin-resistant *Staphylococcus aureus* are challenging problems in health care institutions worldwide. High-level vancomycin resistance is conferred by acquiring either transposable elements of the *VanA* or *VanB* type. *Enterococcus faecalis* $VanY_B$ in the *VanB*-type operon is a D,D-carboxypeptidase that recognizes the peptidyl-D-Ala^4-D-Ala^5 extremity of peptidoglycan and hydrolyses the terminal D-Ala on the extracellular side of the cell wall, thereby increasing the level of glycopeptide antibiotics resistance. However, at the molecular level, it remains unclear how $VanY_B$ manipulates peptidoglycan peptides for vancomycin resistance. In this study, we have determined the crystal structures of *E. faecalis* $VanY_B$ in the D-Ala-D-Ala-bound, D-Ala-bound, and -unbound states. The interactions between $VanY_B$ and D-Ala-D-Ala observed in the crystal provide the molecular basis for the recognition of peptidoglycan substrates by $VanY_B$. Moreover, comparisons with the related VanX and VanXY enzymes reveal distinct structural features of *E. faecalis* $VanY_B$ around the active-site cleft, thus shedding light on its unique substrate specificity. Our results could serve as the foundation for unravelling the molecular mechanism of vancomycin resistance and for developing novel antibiotics against the vancomycin-resistant *Enterococcus* species.

1. Introduction

Enterococcus is a genus of Gram-positive bacteria naturally found in the mammalian gastrointestinal tract [1]. Enterococci are opportunistic pathogens with tolerance to various environmental conditions including extremes of temperature and pH, high salinity, detergents, and antibiotics [2]. Among several dozens of *Enterococcus* species identified, only *Enterococcus faecalis* and *Enterococcus faecium* have been known to be responsible for major human infections, accounting for 90–95% and 5–10% of all the clinical cases, respectively [3]. Although enterococci infection has been considered as an important cause of endocarditis for over a century, it has drawn fresh attentions as a major causative agent of hospital-acquired "nosocomial" urinary tract and wound infections in the last few decades [4]. Unfortunately, not only the intrinsic resistance of *Enterococcus* against many commonly used antibiotics but also its ability to acquire further resistance to other drugs through mutations and transposon elements have been major obstacles to appropriate treatments [2].

Vancomycin is a glycopeptide antibiotic that had been widely used for nearly half a century [5,6]. However, the emergence of vancomycin resistance became particularly problematic [7], since it was used as one of the last resorts in treating serious Gram-positive bacterial infections [8]. Vancomycin binds to the D-alanyl-D-alanine (D-Ala-D-Ala) moiety of the uncross-linked UDP-*N*-acetylmuramyl-L-Ala^1-D-γ-Glu^2-L-Lys^3-D-Ala^4-D-Ala^5 (UDP-NAM-pentapeptide) peptidoglycan precursor at the cell surface with high affinity, thus effectively blocking the formation of cross-linking by transpeptidases and the addition of newly synthesized precursors to the nascent peptidoglycan chain [5]. Unfortunately, some enterococci have acquired vancomycin resistance

Abbreviations: VRE, vancomycin-resistant *Enterococcus*; NAM, *N*-acetylmuramic acid; NAG, *N*-acetylglucosamine; PEG, polyethylene glycol; SAD, single-wavelength anomalous diffraction; RMSD, root-mean-square deviation; SEC-MALS, size-exclusion chromatography with multi-angle light scattering; PDB, Protein Data Bank.

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by modifying the D-Ala-D-Ala moiety of the UDP-NAM-pentapeptide and these enterococci are called vancomycin-resistant enterococci (VRE).

Several types of operons related to this vancomycin resistance have been identified in enterococci. *VanA*, *-B*, *-D*, and *-M* types encode enzymes responsible for replacing the C-terminal dipeptide to a low-affinity moiety, D-alanyl-D-lactate (D-Ala-D-Lac), while *VanC*, *-E*, *-G*, *-L*, and *-N* types encode enzymes that convert the C-terminal dipeptide to D-alanyl-D-serine [9–12]. *E. faecalis* and *E. faecium* harbor the *VanB*-type operon in its chromosome or on independent plasmids, eliciting up to 1000-fold increase in vancomycin resistance [13,14]. The *VanB* operon contains genes encoding the followings: (i) a two-component regulatory system ($VanR_B$ and $VanS_B$) that senses vancomycin and induces the downstream genes, (ii) a dehydrogenase ($VanH_B$) that converts pyruvate to D-Lac, (iii) a ligase (VanB) that covalently conjugates D-Lac to D-Ala, (iv) an cytoplasmic D,D-dipeptidase ($VanX_B$), (v) a membrane-associated D,D-carboxypeptidase ($VanY_B$), and (vi) two proteins of yet unknown functions (VanV and VanW), where the subscripted B denotes that the protein is from the *VanB*-type operon [15–18].

While the main set of enzymes involved in vancomycin resistance are aimed at detecting glycopeptide antibiotics for subsequent intracellular *de novo* synthesis of the modified NAM-L-Ala^1-D-γ-Glu^2-L-Lys^3-D-Ala^4-D-Lac^5 pentapeptide, VanX and VanY facilitate the depletion of vancomycin-susceptible D-Ala-ending precursors. VanX facilitates the hydrolysis of cytoplasmic D-Ala-D-Ala dipeptides produced by an endogenous D-Ala-D-Ala ligase with high specificity [19,20]. VanY recognizes the D-Ala^4-D-Ala^5 moiety of UDP-NAM-pentapeptide on the extracellular side of the cell wall and cleaves the terminal D-Ala^5 [21,22]. In the case of *Enterococcus* species that achieve vancomycin resistance through a D-Ala-D-Ser moiety, VanXY has been expected to elicit dual substrate specificities for both dipeptide and pentapeptide as shown with $VanXY_C$ [23,24], while $VanXY_G$ is deficient in the D,D-pentapeptidase activity [25]. VanX, $VanXY_C$, and $VanXY_G$ have been structurally characterized and structural analyses revealed the characteristic zinc-dependent D,D-peptidase fold and the molecular determinants for selecting substrate precursors [26,27].

Along with VanX D,D-dipeptidase and VanXY D,D-carboxypeptidase, VanY is predicted to be a zinc-dependent D,D-carboxypeptidase in the metallopeptidase family M15, according to the MEROPS database for peptidase resources [28]. Despite the potential significance of $VanY_B$ as a D,D-carboxypeptidase solely existing in the *VanB*-type operon and as a novel anti-VRE drug target, structural information on VanY enzymes has not been available, which limits our comprehensive understanding on substrate specificities of VRE-related D,D-carboxypeptidases. $VanY_B$ is considered as the most important among four known VanY orthologs ($VanY_B$, $VanY_A$, $VanY_M$, and $VanY_D$), since the *VanB*-type operon is predominantly found in the pathogenic *E. faecalis* V583 strain. $VanY_B$ from *E. faecalis* has been shown to exhibit the D,D-carboxypeptidase activity against the peptidoglycan-derived pentapeptides [15]. To elucidate the structural basis of the subtle substrate specificities of these unique metalloenzymes, the overall structural-fold comparison is not enough but elaborate structural analyses of their additionally appended elements and active sites are required.

To provide a structural basis for better understanding of the role of VanY on the modification of peptidoglycan peptides in *Enterococci* vancomycin resistance, here we report the crystal structures of $VanY_B$ from *E. faecalis* V583 strain, as a representative enzyme in the VanY family, in complex with and without the D-Ala-D-Ala dipeptide or D-Ala for comparison. Intensive structural analyses of $VanY_B$ with the related VanX and VanXY enzymes reveal distinct features of $VanY_B$ around the active-site cleft, thus shedding light on the unique substrate specificity of $VanY_B$. Our results could serve as the foundation for the discovery of $VanY_B$ inhibitors as novel antibiotics against VRE.

2. Materials and methods

2.1. Cloning, protein expression, and purification

The TMHMM server [29] predicts that $VanY_B$ from *E. faecalis* V583 contains a transmembrane region (residues Ala25-Val46). Therefore, we overexpressed the recombinant $VanY_B$ lacking the predicted transmembrane region. The *$vanY_B$* gene (EF2297) was PCR-amplified and cloned into the pET-21a(+) vector (Novagen) using *Nhe*I and *Xho*I restriction enzymes. The resulting recombinant $VanY_B$ encompassing residues Thr52-Lys268 is fused with a His_6-containing tag at its C-terminus. It was overexpressed in *Escherichia coli* Rosetta2 (DE3) strain. $VanY_B$ was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 18 °C for 22 h using Luria Broth culture medium supplemented with 1 mM zinc chloride. The harvested cell pellet was resuspended in the lysis buffer containing 20 mM Tris–HCl (pH 7.9), 500 mM sodium chloride, 5 mM imidazole, 1 mM zinc chloride, 5% (w/v) glycerol, and 0.5 mM phenylmethanesulfonyl fluoride, before disruption by French press. The cell lysate was centrifuged at 35,000*g* for 50 min at 4 °C, then filtered through 0.22 μm filter to remove cell debris and any aggregated proteins. The filtrate was loaded onto a HiTrap Chelating HP column (GE Healthcare), which was previously equilibrated with the lysis buffer, and $VanY_B$ was eluted at 150–220 mM imidazole. The eluent was diluted 5-fold with a buffer containing 20 mM Tris–HCl (pH 8.8) before further purification on a HiTrap Q HP column (GE Healthcare). The fractions containing $VanY_B$ were applied to a HiLoad 16/600 Superdex 200 prep grade column (GE Healthcare), which was pre-equilibrated with a buffer containing 20 mM Tris–HCl (pH 7.2) and 200 mM sodium chloride. The purified $VanY_B$ was concentrated to 4.6 mg mL^{-1} for crystallization. The purification steps for Se-Met substituted $VanY_B$ were as above except that the buffer for the final size exclusion chromatography was at pH 7.9.

2.2. Crystallization, data collection, and structure determination

To solve the phase problem by anomalous diffraction, crystals of SeMet-substituted $VanY_B$ were obtained at 23 °C by the sitting-drop vapor diffusion method. Each sitting drop, prepared by mixing equal volumes (1 μL) of the purified protein and the reservoir solution [24% (w/v) polyethylene glycol (PEG) 1500 and 20% (w/v) glycerol], was equilibrated against 50 μL of the reservoir solution. Crystals grown in the sitting drop were already cryoprotected with the reservoir solution and flash-frozen in a nitrogen gas stream at 100 K. Single-wavelength anomalous diffraction (SAD) data of the D-Ala-D-Ala-unbound form of $VanY_B$ ($VanY_B$·unbound) were collected to 1.65 Å from a crystal of SeMet-substituted $VanY_B$ (Table 1). Data from native crystals were not collected because they diffracted only up to 3.5 Å. Raw X-ray diffraction data were processed and scaled using the *HKL-2000* program suite [30]. SAD phases were initially calculated with *AUTOSOL* in the *PHENIX* software suite [31] and further improved by the automatic model building program *RESOLVE* [32], resulting in an initial model. The initial model was further refined to the final model using iterative cycles of model building with *Coot* [33] and subsequent refinement with *Refmac5* in the *CCP4* suite [34,35].

X-ray diffraction data for the D-Ala-D-Ala-bound form ($VanY_B$·dipeptide) were collected at 100 K after the SeMet crystal was pre-incubated for 2 min in a cryoprotectant solution containing 5.4 mM D-Ala-D-Ala dipeptide and 2 mM $CuCl_2$ for the suppression of the D,D-peptidase activity. X-ray diffraction data for the D-Ala-bound form ($VanY_B$·D-Ala) were collected at 100 K after quick soaking crystals for 5 min in a cryoprotectant solution [0.1 M sodium acetate, 30% (w/v) PEG 1500, and 25% (w/v) glycerol] containing 100 mM L-Ala^1-D-γ-Glu^2-L-Lys^3-D-Ala^4-D-Ala^5 pentapeptide as a substrate (Sigma-Aldrich), only the D-Ala^5 part of which was shown in the refined structure, as an enzymatic product, likely due to the cleavage reaction by $VanY_B$ in crystals.