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Purification and characterization of the VanB ligase associated with type B vancomycin resistance in *Enterococcus faecalis* V583

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Abstract Acquired resistance to glycopeptides in enterococci is associated with the production of D-Alanine:D-Alanine ligase-related proteins. The VanA protein associated with high-level vancomycin and teicoplanin resistance (VanA phenotype) synthesizes a new peptidoglycan precursor, D-alanine-D-lactate, that has reduced glycopeptide affinity. Production of a similar protein, VanB, is induced in strains that display variable levels of vancomycin resistance but remain susceptible to teicoplanin (VanB phenotype). This paper describes the over-production, purification and characterization of VanB. Comparison of kinetic parameters of the two Van enzymes suggests that differences in catalytic efficiency could account, at least in part, for the various levels of vancomycin resistance.

Key words: D-Ala:D-Ala ligase; Glycopeptide; Vancomycin; Depsipeptide; Peptidoglycan; *Enterococcus*

1. Introduction

The C-terminal D-Ala-D-Ala residues of bacterial cell wall precursors are the target for glycopeptide antibiotics, such as vancomycin and teicoplanin, which inhibit the transglycosylation and transpeptidation reactions of peptidoglycan assembly [15]. Three decades after introduction of vancomycin in human therapy the first resistant clinical isolates of *Enterococcus* were detected [11].

In enterococci at least one protein is produced in association with one of the three glycopeptide resistance phenotypes (VanA, VanB and VanC) [1]. Isolates with high-level resistance to vancomycin (MIC from 64 to 1,000 μg/ml) and to teicoplanin (MIC from 16 to 512 μg/ml) belong to the VanA phenotype. This inducible resistance is associated with production of the VanA protein, a D-Ala:D-Ala ligase of altered substrate specificity which preferentially catalyzes ester bond formation between D-Ala and a D-2-hydroxyacid [5,6]. D-Lactate (D-Lac) was identified as the in vivo substrate for VanA [2,10]. The depsipeptide D-Ala-D-Lac synthesized by VanA is incorporated into the cell wall by the usual pathway of peptidoglycan biosynthesis. The low affinity of glycopeptides for the modified cell wall precursors allows peptidoglycan synthesis in the presence of the antibiotics [1]. In addition to VanA, four proteins are required for inducible vancomycin resistance: the VanH dehydrogenase for synthesis of D-Lac [1]; VanX, a dipeptidase which hydrolyses D-Ala-D-Ala [16]; and VanR-VanS, a two-component regulatory system [3,19]. Strains of *E. faecium* and *E. faecalis* which display variable levels of inducible resistance to vancomycin (MIC from 4 to 1,000 μg/ml) and retain susceptibility to teicoplanin (MIC < 2 μg/ml) are of the VanB type [14,18]. The VanC phenotype corresponds to constitutive low-level resistance to vancomycin alone (MIC from 2 to 32 μg/ml) and is found in *E. casseliflavus*, *E. flavescens* and *E. gallinarum* [8,12]. The *vanB* and *vanC* genes encode two proteins, VanB and VanC, that are structurally related to VanA and to D-Ala:D-Ala ligases [8,9]. Analysis of the peptidoglycan composition of VanB and VanC type strains allowed identification of D-Lac and D-serine, respectively, as C-terminal residues of peptidoglycan precursors [4,9,17]. These observations imply that VanB and VanC are also D-Ala:D-Ala ligases of altered substrate specificity.

Since differences in specificity and catalytic efficiency of the Van proteins could account for the three phenotypes detected so far, we compared the kinetic parameters of the purified proteins. In this report, we describe overproduction, purification and kinetic characterization of the VanB protein of *E. faecalis* V583.

2. Experimental

2.1. Construction of plasmid pAT204 for over-production of the VanB protein

The 3.3-kb *Kpn*I–*Hin*dIII insert of pAT202 [9], containing the *vanB* gene of *E. faecalis* V583, was cloned into the *Sma*I site of pUC19 using a commercial DNA blunting kit (Amersham). A plasmid with *vanB* and the *lac* promoter in the same orientation that allows control of *vanB* expression was selected for further studies. Two deletions were introduced to prevent expression of the genes flanking *vanB*: (i) the region upstream from *vanB* was deleted by exonuclease III–S1 nuclease digestion (Erase-a-Base, Promega) leaving 244 bp upstream from the ATG start codon: (ii) the fragment between the *Ssp*I site 288 bp downstream from the TGA stop codon and the unique *Ssp*I site of pUC19 was excised to remove the region downstream from *vanB*. The resulting plasmid, designated pAT204, allows expression of the *vanB* gene under control of the *lac* promoter.

2.2. Over-production and purification of VanB

All the purification steps were performed at 4°C. Enzyme activity was monitored by thin-layer chromatographic assay [5] or, when possible, by the ADP release coupled assay [7]. Protein concentration was determined by measurement of the absorbance at 280 nm.

Plasmid pAT204 was transformed into *Escherichia coli* HB101 and a transformant was grown at 37°C in 5 l of Luria broth (LB) containing ampicillin (100 μg/ml) to an absorbance of 0.6 at 600 nm. Production of VanB was induced by addition of isopropylthiogalactoside to a final concentration of 1 mM. Cells were then grown for 3 h and harvested

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by centrifugation at 8000 × g for 15 min. The cell pellet (20 g) was suspended in 50 ml of 50 mM HEPES (pH 7.5) containing 5 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT and was disrupted with a Branson Sonifier-250 sonicator. Cell debris were removed by centrifugation at 25,000 × g for 30 min.

The supernatant was loaded onto a Q-sepharose Fast-flow column (50 ml) (Pharmacia) equilibrated with HEPES 50 mM (pH 7.5) containing 5 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT. The column was eluted in two steps, first at 100 mM and then at 300 mM NaCl. VanB was recovered at 300 mM. The active fractions were pooled and concentrated using an ultrafiltration device (Novacell, Filtron) equipped with a 10K membrane. The concentrated pool (10 ml) was passed twice through a 2 ml ATP-OH ribose attachment-agarose column (Sigma) at a flow rate of 25 ml/h, until no ATP-ase activity was detected in the solution using the ADP release coupled assay [7] without addition of D-alanine.

2.3. Substrate specificity and kinetic analysis of VanB

Substrate specificity was assayed by thin-layer chromatographic (TLC) assay [6]. The kinetic parameters of VanB for D-Ala:D-Ala ligase activity were determined using the equation:

$$v = \frac{V_m[A]^2}{K_{A_1}K_{A_2} + K_{A_2}[A] + [A]^2} \quad (1)$$

previously established by Neuhaus [13] where K_{A_1} and K_{A_2} are the Michaelis constants of, respectively, the first (N-terminal) and the second (C-terminal) alanine residues, and [A] is D-Ala concentration. K_m values were calculated by fitting the data obtained by the spectrophotometric assay described by Daub et al. [7] to the above equation using the Enzfitter programme. Kinetic parameters for the D-Ala:D-2-hydroxyacid synthase activity were determined at 5 mM of D-Ala. D-Ala-D-hydroxyacid was separated from D-Ala-D-Ala by TLC on cellulose using 1-[^{14}C]D-Ala (40 mCi/mmol) and the amount of the depsipeptide formed was determined by radioactivity counting. In the case of D-Ala-D-Lac synthesis, 1-[^{14}C]D-lactate (50 mCi/mmol) was used instead of 1-[^{14}C]D-Ala since D-Ala-D-Lac co-migrates with D-Ala-D-Ala in the assay conditions [6]. The K_m for D-hydroxyacid was calculated according to the equation:

$$v = \frac{V_m}{1 + \frac{K_B}{[B]}\left\{\frac{K_{A_1}}{[A]} + \frac{[A]}{K_{A_2}} + 1\right\}} \quad (2)$$

as described by Neuhaus [13], where K_{A_1} and K_{A_2} have the same significance given in eq. 1; K_B and [B] are, respectively, the Michaelis constant and concentration of the D-hydroxyacid under assay. ATP was used at saturating concentration of 10 mM.

Table 1
Kinetic properties of VanB and VanA as D-Ala-D-Ala ligases

Substrate/product		VanB	VanA[a]
D-Ala-D-Ala	K_{A_1} (mM)	1.2 ± 0.4[b]	3.4
	K_{A_2} (mM)	34 ± 1.1	38
	k_{cat} (min^{-1})	246 ± 2.0	295
	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	120 ± 4.1	129
ATP	K_m (μM)	69 ± 2.1	116

K_{A_1} and K_{A_2} refer to the Michaelis constants for the binding of, respectively, the first (N-terminal) and second (C-terminal) residues.
[a] Data from [5].
[b] Mean of three determinations.

3. Results and discussion

The VanB protein was over-produced to ca. 10% of the soluble proteins of *E. coli* HB101 harboring recombinant plasmid pAT204. The enzyme was detected during purification by its ability to synthesize D-Ala-D-2-hydroxybutyrate by TLC. A two-step purification of VanB, including Q-Sepharose anion-exchange chromatography and ATP-agarose affinity chromatography, was devised. Stepwise elutions at 100 mM and 300 mM NaCl from Q-Sepharose allowed vanB protein recovery in the high salt eluent. Unlike VanA [5], VanB was found to aggregate upon ammonium sulfate fractionation, suggesting a difference in hydrophobic surface pattern. ATP-agarose chromatography was finally used to eliminate contaminating ATPase activity which interfered with the D-Ala:D-Ala ligase ADP release coupled assay. SDS-PAGE displayed a single band at 38 kDa (data not shown). Starting from 20 g of wet cells, this two-step purification procedure yielded 30 mg of VanB protein with a specific activity of 11 U/mg. A fraction of the ATP-agarose eluate was subjected to sequence analysis. The N-terminal sequence (M-N-K-I-K-V-A-I-I-F-G) determined on 11 Edman degradation cycles was identical with that deduced from the nucleotide sequence [9]. No contaminating sequence could be detected from HPLC analysis of PTH-amino acids, suggesting a high degree of protein purity.

Like VanA [5,6], VanB was found to possess D-Ala:D-Ala

Table 2
Kinetic properties of VanB and VanA as D-Ala-D-2-hydroxyacid ligases

D-Ala-D-X		VanB	VanA[a]
D-Ala-D-2-hydroxybutyrate	K_{A_1} (mM)	nd[b]	4.1
	K_B (mM)	3.0 ± 0.28[d]	0.6 (0.5 ± 0.09)[c]
	k_{cat} (min^{-1})	15 ± 1.2	108 (78 ± 8.6)
	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	83.6 ± 16.1	3000 (2866 ± 308)
D-Ala-D-2-hydroxyvalerate	K_{A_1} (mM)	nd	nd
	K_B (mM)	8.3 ± 0.26	2.3
	k_{cat} (min^{-1})	25 ± 2.5	156
	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	50.2 ± 5.3	810
D-Ala-D-lactate	K_{A_1} (mM)	nd	3.2
	K_B (mM)	11.4 ± 1	7.1
	k_{cat} (min^{-1})	28.2 ± 1.6	94
	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	41.4 ± 2.4	220

K_{A_1} and K_B refer to the Michaelis constants for the binding of, respectively, D-Ala and D-hydroxyacid.
[a] Data from [5].
[b] nd = not determined.
[c] Values in parentheses are calculated with purified VanA from *E. coli* HB101/pAT219 (Dutka-Malen et al., unpublished).
[d] Mean of three determinations.

ligase activity. Despite slight differences in K_m and k_{cat} values, the catalytic efficiencies of the two enzymes for D-Ala-D-Ala synthesis were similar (Table 1). The substrate specificity of VanB for amino acids was studied by TLC analysis of radioactive dipeptides produced following incubation of [^{14}C]D-Ala (0.2 mM) with 10 mM of the amino acid. Qualitatively, VanB was able to synthesize mixed dipeptides with hydrophobic D-amino acids such as D-phenylalanine, D-methionine, D-valine, D-neurleucine, D-isoleucine. K_m values of VanB for D-hydroxyacids were determined at saturating concentrations of D-Ala and ATP, respectively, 5 and 10 mM, and were found to be 2- to 5-fold higher (Table 2) than those of VanA [6]. D-2-Hydroxybutyrate was the best substrate with a K_m of 3 mM, lower than those for D-Lac (11.4 mM) and D-2-hydroxyvalerate (8.3 mM). As found for VanA [6], these results indicate the longer side chain preference of VanB. Furthermore, catalytic efficiencies (k_{cat}/K_m) of the D-hydroxyacids tested for VanB were 5- to 35-times lower than those for VanA (Table 2).

Our results demonstrate that VanB is a depsipeptide synthase displaying a substrate specificity similar to that of VanA [5,6]. This is not surprising given the extensive similarity (76% amino acid identity) between VanA and VanB [9] and the identification of D-Lac as in vivo substrate for both enzymes [9,10]. The catalytic efficiency for depsipeptide synthesis of VanB is lower than that of VanA (Table 2). Therefore, low-level resistance to vancomycin of *E. faecalis* V583 (MIC = 64 μg/ml) might be related to synthesis of smaller amounts of cell wall precursors terminating in D-Lac. However, a lower level of production or activity of other enzymes required for resistance (D-2-hydroxyacid dehydrogenase, dipeptidase), as well as a lower VanB concentration, could also contribute to the difference in expression of vancomycin susceptibility. Finally, a difference in specificity between the two-component regulatory systems for expression of the *vanA* [3] and *vanB* (Evers et al., unpublished data) gene clusters could account for the fact that VanB strains remain susceptible to teicoplanin, since this antibiotic is not an inducer.

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