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Characterization of VanY, a DD-Carboxypeptidase from Vancomycin-Resistant *Enterococcus faecium* BM4147

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VanY is a protein with a molecular mass of 34.8 kDa encoded by *vanY*, a member of the high-level vancomycin resistance gene cluster found on plasmid pIP816 in *Enterococcus faecium* BM4147. Extracts from *Escherichia coli* JM83 bearing plasmid pAT383, which contains the *vanY* gene, were examined for enzymatic hydrolysis of peptidoglycan precursors. VanY was associated with the cell membranes and cleaved the C-terminal D-alanine residue of UDP-muramyl-pentapeptide but did not display transpeptidase or β-lactamase activities. The DD-carboxypeptidase activity was not inhibited by β-lactam antibiotics. VanY released the C-terminal D-hydroxy acid from depsipeptides produced by the vancomycin resistance protein VanA. These results demonstrate that VanY should contribute in vivo to the hydrolysis of both the D-alanyl–D-alanine- and the depsipeptide-containing peptidoglycan precursors.

Enterococci have developed resistance to virtually all clinically useful antibiotics (14). Recently, resistance of enterococci to the glycopeptides vancomycin and teicoplanin has become a serious clinical problem. We have previously demonstrated that vancomycin resistance in *Enterococcus faecium* BM4147 requires two proteins, VanA and VanH, which are a D-Ala–D-Ala ligase of altered specificity and a 2-keto acid dehydrogenase, respectively (5, 6). These enzymes act in concert in the formation of a depsipeptide with the form D-Ala–O–D-X which can be incorporated into peptidoglycan precursors. This would result in the biosynthesis of muropeptides terminating in a depsipeptide, probably D-Ala–D-lactate (2), rather than the ubiquitous D-Ala–D-Ala C terminus. Vancomycin acts by binding to *N*-acyl-D-Ala–D-Ala and consequently preventing the transglycosylation and transpeptidation steps of peptidoglycan assembly (16). We (6) and others (15) have previously demonstrated that vancomycin has little or no affinity for depsipeptides, thereby providing the rationale for resistance in cells containing the *vanA* and *vanH* genes.

Evidence for a glycopeptide-inducible DD-carboxypeptidase in extracts of vancomycin-resistant enterococcal strains has been presented previously (1, 10). It has been proposed that the action of a DD-carboxypeptidase could result in the decreased availability of D-Ala–D-Ala termini and could therefore provide a component of the mechanism for glycopeptide resistance by effectively removing the antibiotic target from the cell surface. VanY is a protein encoded by the *van* gene cluster of pIP816, the plasmid that confers high-level resistance to vancomycin in *E. faecium* BM4147 (4). VanY has been cloned into *Escherichia coli* JM83, and primary sequence analysis shows no significant similarity with known DD-carboxypeptidases (4). In this report, we identify VanY as a DD-carboxypeptidase and a DD-carboxyesterase which is active on precursors synthesized by VanH and VanA.

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MATERIALS AND METHODS

Bacterial strains, cell growth, and preparation of VanY. The identification, cloning, and sequencing of the *vanY* gene, which is located downstream from *vanX* in plasmid pIP816 (3), will be reported elsewhere (4). *E. coli* JM83 and *E. coli* JM83/pAT383 were used in these studies. The plasmid pAT383 bears the *vanY* gene under the control of the *lac* promoter of plasmid pAT114 (4); pAT383 does not encode a β-lactamase and was maintained by the addition of 0.05 mg of kanamycin per ml to the cultures. VanY production was induced by the addition of 10 ml of 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to a 1-liter culture of JM83/pAT383 in Luria broth when the cell density reached an optical density at 595 nm of 0.55. Cells were grown for an additional 4 h, harvested, and washed with 0.8% (wt/vol) NaCl. The pellet was resuspended in 25 ml of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–5 mM $MgCl_2$–0.5 M NaCl–1 mM EDTA–1 mM dithiothreitol (pH 7.5) and disrupted by two successive passages through a French pressure cell at 1,100 lb/in^2. Cell debris was removed by centrifugation at 1,000 × *g* for 10 min. The supernatant (S1) was then centrifuged at 110,000 × *g* for 60 min. The supernatant (S2) was collected, and the pellet (cell membrane fraction) was suspended in 5 ml of 50 mM HEPES–5 mM $MgCl_2$–1 mM EDTA–1 mM dithiothreitol–1% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} (pH 7.5) and stirred gently at 4°C for 60 min. The suspension was centrifuged at 110,000 × *g* for 30 min, and the supernatant (S3) was collected.

Pure VanA (5) and *E. coli* D-Ala–D-Ala-adding enzyme (6) were prepared as described previously.

DD-Carboxypeptidase assays. (i) Fluorometric OPTA method (9). Reaction mixtures contained 100 μg of S3 protein, 5 mM $Zn(OAc)_2$, 10 mM diacetyl-L-Lys-D-Ala–D-Ala, and 50 mM $NaHCO_3$ (pH 8.5) in a final volume of 20 μl. The reaction was quenched by the addition of 5 μl of 250 mM HCl followed by the addition of 75 μl of water. Enzymatically released D-Ala was monitored by the addition of 100 μl of Fluoraldehyde (*o*-phthaldialdehyde [OPTA]) solution (Pierce) followed by incubation at room temperature for 5

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min. Water (0.8 ml) was added, 10 μl was removed and diluted 100-fold, and the fluorescence was monitored (λex, 340 nm; λem, 455 nm). Assays were quantitated from a standard curve prepared with standard D-Ala.

(ii) High-pressure liquid chromatography (HPLC) assay. UDP-MurNAc-L-Ala-D-Glu-mDAP-D-Ala-D-Ala (10 nmol) was incubated with 100 μg of protein as described above (omitting the tripeptide). The reaction was quenched by rapid heating to 70°C, and the reaction mixture was injected onto a Vydac C18 column (250 by 4 mm) equilibrated with 50 mM NH_4OCOH (pH 4.5) at a flow rate of 0.5 ml/min. UDP-MurNAc-peptides were monitored at 262 nm and quantitated by integration.

(iii) TLC assay. ^{14}C-labeled peptides and depsipeptides were prepared by incubation of VanA with either [^{14}C]D-Ala or [^{14}C]D-lactate which was prepared by reduction of [^{14}C]pyruvate with D-lactate dehydrogenase. Incubation of the dipeptide or depsipeptide with UDP-MurNAc-L-Ala-D-Glu-mDAP- and D-Ala-D-Ala-adding enzyme was followed by purification by HPLC with 50 mM NH_4OCOH (pH 4.5) (6), which afforded the compounds UDP-MurNAc-L-Ala-D-Glu-mDAP-[^{14}C]D-Ala-[^{14}C]D-Ala and UDP-MurNAc-L-Ala-D-Glu-mDAP-D-Ala-[^{14}C]D-lactate. Labeled muropeptide or depsipeptide was incubated with 100 μg of S3 protein as described above, and the reaction was quenched by immersion in an ice bath. A 10-μl sample was applied to a cellulose thin-layer chromatography (TLC) plate and developed with *n*-butanol-acetic acid-water (12:3:5). Plates were then air dried and subjected to autoradiography. Spots corresponding to D-Ala or D-lactate and UDP-muramyl-peptide were cut out and analyzed by liquid scintillation counting.

Transpeptidase assay. UDP-MurNAc-pentapeptide (1 nmol) was incubated as described above in the presence of [^{14}C]glycine (0.4 nmol) or [^{14}C]D-Ala (0.6 nmol) at 37°C for 4 h. The sample was subjected to reverse-phase HPLC as described above; 1-min fractions were collected and analyzed by liquid scintillation counting.

β-Lactamase assay. The iodometric method of Frère et al. (8) was followed. Protein (100 μg) was added to a solution of penicillin G (10 mM) in 5 mM $Zn(OAc)_2$-50 mM $NaHCO_3$ (pH 8.5) in a final volume of 20 μl. The reaction was carried out for 2 h at 37°C and quenched by the addition of 200 μl of 1 M Na acetate (pH 3.5), which was followed by that of 200 μl of a 1:1 (vol/vol) solution of 0.8% starch and 4.8 mM KI-0.24 mM I_2. The A_{620} was monitored after a 10-min incubation at room temperature.

RESULTS

DD-Carboxypeptidase activity encoded by *vanY* in *E. coli* JM83/pAT383. DD-Carboxypeptidase activity was located primarily in the membrane fractions of *E. coli* JM83/pAT383 and absent in *E. coli* JM83. We experienced some difficulty in obtaining precise activity measurements in crude enzyme fractions which could be alleviated by solubilization of the DD-carboxypeptidase activity with detergents. Sodium lauryl sarcosine, CHAPS, and Triton X-100 were examined, and only the latter two were able to solubilize active VanY; therefore, all further work was done with CHAPS-solubilized membrane proteins. DD-Carboxypeptidase activity was recovered primarily in the detergent-extracted cell membranes of *E. coli* JM83/pAT383, and no detectable activity (determined by the OPTA method) was associated with either the membrane or soluble fraction of *E. coli* JM83 (Table 1). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of CHAPS-solubilized membrane proteins indicated a band of ≈35 kDa which is predicted from the deduced primary sequence of VanY (4) (Fig. 1).

TABLE 1. Localization of VanY activity in *E. coli* JM83 extracts

Strain and fraction(s)	DD-Carboxypeptidase activity[a] (nmoles of D-Ala)
JM83	
Cytosolic fraction (S2)	ND[b]
Solubilized membranes (S3)	ND[b]
JM83/pAT383	
Cytosolic fraction (S2)	252
Solubilized membranes (S3)	474

[a] Determined by using the OPTA method with 10 mM diacetyl-Lys-D-Ala-D-Ala incubated with 0.1 mg of protein at 37°C for 1 h.
[b] No detectable activity (<0.2 nmol).

In order to confirm that VanY was indeed a DD-carboxypeptidase and not a nonspecific protease, we sought to identify the tetrapeptide product of the reaction. Incubation of CHAPS-solubilized membrane proteins with UDP-MurNAc-pentapeptide followed by reverse-phase HPLC analysis resulted in a decrease in the peak corresponding to pentapeptide (retention time = 24 to 25 min) with a concomitant increase in a new peak at 14 to 15 min. This peak was tentatively assigned to UDP-MurNAc-tetrapeptide (UDP-MurNAc-tripeptide standard eluted at 9 to 10 min) (Fig. 2C) and was observed only with JM83/pAT383 (Table 2). The assignment of the new peak at 14 to 15 min to UDP-MurNAc-tetrapeptide was confirmed by incubation of JM83/pAT383 S3 fractions with UDP-MurNAc-L-Ala-D-Glu-mDAP-[^{14}C]D-Ala-[^{14}C]D-Ala followed by HPLC. The results of this experiment (Fig. 3) indicate loss of the peak at 25 min (pentapeptide) and distribution of radioactivity into two new peaks at 7 to 8 min and 14 to 15 min which correspond to [^{14}C]D-Ala and UDP-MurNAc-L-Ala-D-Glu-mDAP-[^{14}C]D-Ala, respectively. No evidence for LD-carboxypeptidase activity (change in the UDP-muramyl-tripeptide over time) was observed by using HPLC.

DD-Carboxyesterase activity. Incubation of CHAPS-solubi-

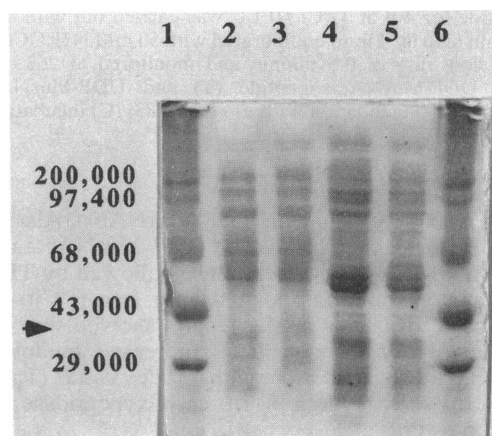

FIG. 1. SDS-PAGE of *E. coli* JM83 proteins. The gel (11%) was stained with Coomassie blue. Lanes: 1 and 6, molecular weight markers; 2, *E. coli* JM83/pAT383 S3; 3, *E. coli* JM83 S3; 4, *E. coli* JM83/pAT383 S2; 5, *E. coli* JM83 S2. The arrow indicates 35 kDa.

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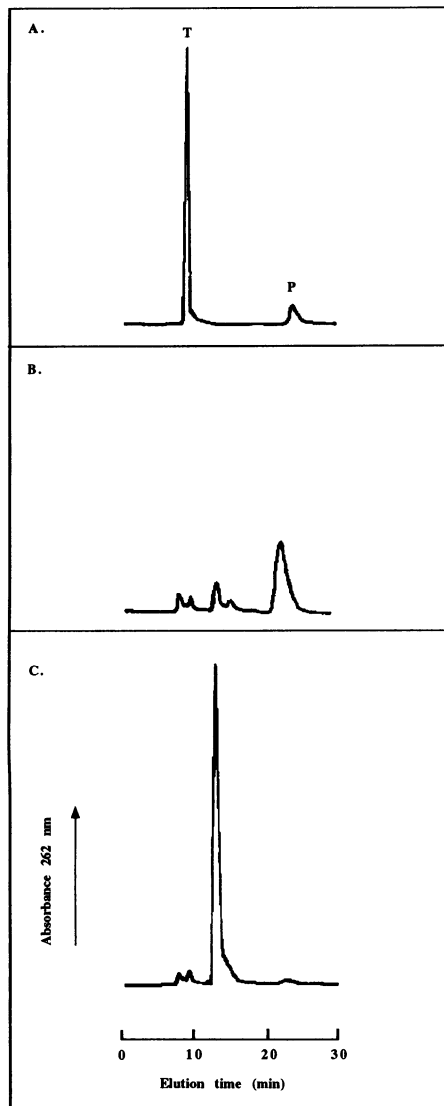

FIG. 2. Fate of UDP-MurNAc-pentapeptide incubated with *E. coli* detergent-extracted membrane proteins. Reactions were carried out with 0.1 mg of S3 protein and 10 nmol of UDP-MurNAc-pentapeptide for 4 h at 37°C. HPLC was carried out with a Vydac C18 column (250 by 4 mm) equilibrated with 50 mM NH_4OCOH (pH 4.5) at a flow rate of 0.5 ml/min and monitored at 262 nm. (A) Standard UDP-MurNAc-tripeptide (T) and UDP-MurNAc-pentapeptide (P); (B) incubation with *E. coli* JM83; (C) incubation with *E. coli* JM83/pAT383.

lized membrane proteins with UDP–MurNAc–L-Ala–D-Glu–mDAP–[^{14}C]D-Ala–[^{14}C]D-Ala and UDP–MurNAc–L-Ala–D-Glu–mDAP–D-Ala–[^{14}C]D-lactate was followed by TLC and quantitation of counts associated with free [^{14}C]D-Ala or [^{14}C]D-lactate. Analysis of the results showed that the release of D-Ala or D-lactate was increased fivefold over background after incubation with crude VanY (Table 3). Therefore, VanY is both a DD-carboxypeptidase and a DD-carboxyesterase.

Effects of β-lactams on VanY activity. The effect of a number of β-lactams on VanY DD-carboxypeptidase activity was examined. VanY activity was impervious to penicillin G, ampicillin, and cephalosporin C at concentrations of 10

TABLE 2. HPLC determination of VanY activity on UDP-MurNAc-pentapeptide

Strain and trial no.	Area under the peak (% total integrated area)[a]	
	Tetrapeptide	Pentapeptide
JM83		
1	11	69
2	12	63
JM83/pAT383		
1	91	2
2	73	2

[a] UDP–MurNAc–L-Ala–D-Glu–mDAP–D-Ala–D-Ala (10 nmol) incubated with 0.1 mg of S3 protein at 37°C for 4 h.

mM. Methicillin showed weak inhibition (20%) at the same concentration. VanY is therefore not a penicillin-binding protein. The possibility that the observed insensitivity toward β-lactams was due to an intrinsic β-lactamase activity associated with VanY was explored with penicillin G. No evidence for hydrolysis of the antibiotic was detected by using an iodometric method (8).

Transpeptidase. Transpeptidase activity was monitored by using [^{14}C]D-Ala and [^{14}C]Gly as acceptors, and products were then analyzed by HPLC. No radioactivity was associated with the fractions corresponding to the UDP-MurNAc-pentapeptide. Therefore, VanY will only transfer the tetrapeptide to water, i.e., it possesses DD-carboxypeptidase activity exclusively.

DISCUSSION

Vancomycin resistance in *E. faecium* BM4147 has recently been shown to be associated with the synthesis of a depsipeptide cell wall precursor which no longer has the ability to bind the antibiotic (6). High-level resistance to glycopeptides requires five genes, *vanR*, *vanS*, *vanH*, *vanA*,

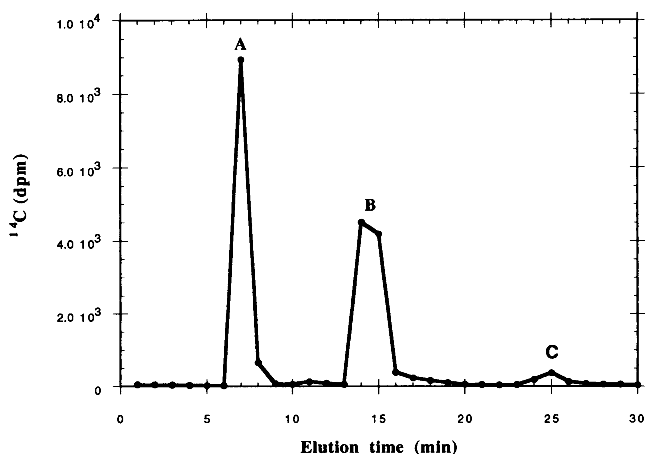

FIG. 3. HPLC of UDP–MurNAc–L-Ala–D-Glu–mDAP–[^{14}C]D-Ala–[^{14}C]D-Ala treated with *E. coli* JM83/pAT383 detergent-extracted membrane proteins. Conditions were as indicated in the legend to Fig. 2 except that UDP–MurNAc–L-Ala–D-Glu–mDAP–[^{14}C]D-Ala–[^{14}C]D-Ala was substituted for unlabeled UDP-MurNAc-pentapeptide. Aliquots (0.5 ml) were collected and added to 10 ml of scintillant before counting. Peak A, D-Ala; peak B, UDP-MurNAc-tetrapeptide; peak C, UDP-MurNAc-pentapeptide.

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TABLE 3. TLC determination of [^{14}C]D-Ala and [^{14}C]D-lactate release by VanY activity on UDP–MurNAc–L-Ala–D-Glu–mDAP–[^{14}C]D-Ala–[^{14}C]D-Ala and UDP–MurNAc–L-Ala–D-Glu–mDAP–D-Ala–[^{14}C]D-lactate

Strain and trial no.	% Total dpm recovered[a]	
	D-Ala fraction	D-Lactate fraction
JM83		
1	12	10
2	10	13
JM83/pAT383		
1	49	56
2	46	49

[a] UDP–MurNAc–L-Ala–D-Glu–mDAP–[^{14}C]D-Ala–[^{14}C]D-Ala (85 pmol) and UDP–MurNAc–L-Ala–D-Glu–mDAP–D-Ala–[^{14}C]D-lactate (90 pmol) were incubated with 0.1 mg of S3 protein for 4 h at 37°C. Results are reported as a percentage because of different total disintegrations per minute (dpm) recoveries between samples; therefore, the percent recovered = dpm in D-X fraction ÷ (dpm in D-X fraction + dpm in UDP-muramyl-peptide fraction).

and *vanX*, associated with plasmid pIP816 (3). An additional open reading frame downstream from *vanX* has been shown to encode a 34.8-kDa protein, VanY, which demonstrates DD-carboxypeptidase activity as tested by a microbiological assay, although it bears no primary structural similarity to penicillin-binding proteins (4).

The present study confirms that *vanY* directs the production of a DD-carboxypeptidase in *E. coli* JM83/pAT383. The enzyme was located principally in the cell membranes, consistent with the presence of a predicted N-terminal membrane-spanning sequence (4), and was solubilized without loss of activity into CHAPS or Triton X-100 micelles. The enzyme activity was shown to be that of a DD-carboxypeptidase by quantitation of the release of D-Ala from the tripeptide substrate diacetyl–L-Lys–D-Ala–D-Ala and by identification of free D-Ala and UDP-MurNAc-tetrapeptide released from UDP-MurNAc-pentapeptide by HPLC and TLC.

In view of the fact that the *van* gene cluster encodes a depsipeptide synthesis system (6), we sought to prove that VanY also possessed DD-carboxyesterase activity. This result was expected, since esters, with a comparatively weak carbonyl-oxygen bond, are more susceptible to nucleophilic attack from water, directly or perhaps from an active-site serine, than the corresponding amide (assuming a similar chemical mechanism with known DD-carboxypeptidases) (7, 11). The release of [^{14}C]D-lactate from UDP–MurNAc–L-Ala–D-Glu–mDAP–D-Ala–[^{14}C]D-lactate by VanY indicated that it is also a DD-carboxyesterase.

VanY shows little sensitivity toward β-lactam antibiotics, a result which is consistent with a recent report on the effects of penicillin on DD-carboxypeptidase activity in membrane fractions from vancomycin-induced *E. faecium* (10). In addition, plasmid pIP816 does not encode any penicillin-binding proteins (13). VanY does not show β-lactamase activity by iodometric assay, indicating that the observed insensitivity to β-lactams is unlikely to be the result of hydrolysis of the antibiotic. These intriguing results await further structural analysis of VanY in order to clarify the molecular basis for the poor interaction with β-lactams. The intrinsic resistance of VanY to β-lactams is notable especially in the context of the well-documented synergism of vancomycin-penicillin combinations against vancomycin-resistant enterococci (12, 17, 18).

An important consideration in the study of DD-carboxypeptidases is their potential for transpeptidase activity, that is, the fact that amines, rather than water, could displace an acyl-enzyme intermediate. No evidence for transpeptidase activity was found by using [^{14}C]D-Ala or [^{14}C]Gly as potential acceptors; therefore, VanY follows the hydrolytic pathway exclusively.

Given the fact that vancomycin-resistant enterococci show DD-carboxypeptidase activity, the physiological role of VanY is of interest. It has been suggested that a possible role for the vancomycin-inducible DD-carboxypeptidase could be to selectively control the abundance of D-Ala–D-Ala-containing peptidoglycan precursors (10). Given the demonstrated vulnerability of depsipeptides to VanY and to DD-carboxypeptidases in general, this hypothesis appears unlikely, as no selectivity toward dipeptides would be predicted. A DD-carboxypeptidase could contribute to vancomycin resistance by hydrolyzing either the terminal D-Ala–D-Ala or D-Ala–D-lactate to give only a tetrapeptide, which does not bind the drug. However, since high-level resistance has been demonstrated to require only the five genes *vanR*, *vanS*, *vanA*, *vanH*, and *vanX* (3), a DD-carboxypeptidase is therefore not essential for glycopeptide resistance. Nonetheless, the location of *vanY* on pIP816 and the reports of a vancomycin-inducible DD-carboxypeptidase in resistant *E. faecium* and *E. faecalis* suggest linkage. Future work in this area will undoubtedly provide insight into the role(s), if any, of VanY in cell wall biosynthesis.

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