Serine and alanine racemase activities of VanT: a protein necessary for vancomycin resistance in *Enterococcus gallinarum* BM4174

Cesar A. Arias,[†] Jan Weisner, Jonathan M. Blackburn and Peter E. Reynolds

Author for correspondence: Cesar A. Arias. Tel: +57 1 633 1512. Fax: +1 917 477 3388.
e-mail: caa22@cable.net.co

Department of
Biochemistry, University of
Cambridge, Tennis Court
Road, The Downing Site,
Cambridge CB2 1QW, UK

Vancomycin resistance in *Enterococcus gallinarum* results from the production of UDP-MurNAc-pentapeptide[D-Ser]. VanT, a membrane-bound serine racemase, is one of three proteins essential for this resistance. To investigate the selectivity of racemization of L-Ser or L-Ala by VanT, a strain of *Escherichia coli* TKL-10 that requires D-Ala for growth at 42 °C was used as host for transformation experiments using plasmids containing the full-length *vanT* from *Ent. gallinarum* or the alanine racemase gene (*alr*) of *Bacillus stearothermophilus*: both plasmids were able to complement *E. coli* TKL-10 at 42 °C. No alanine or serine racemase activities were detected in the host strain *E. coli* TKL-10 grown at 30, 34 or 37 °C. Serine and alanine racemase activities were found almost exclusively (96%) in the membrane fraction of *E. coli* TKL-10/pCA4(*vanT*): the alanine racemase activity of VanT was 14% of the serine racemase activity in both *E. coli* TKL-10/pCA4(*vanT*) and *E. coli* XL-1 Blue/pCA4(*vanT*). Alanine racemase activity was present mainly (95%) in the cytoplasmic fraction of *E. coli* TKL-10/pJW40(*alr*), with a trace (1.6%) of serine racemase activity. Additionally, DNA encoding the soluble domain of VanT was cloned and expressed in *E. coli* M15 as a His-tagged polypeptide and purified: this polypeptide also exhibited both serine and alanine racemase activities; the latter was approximately 18% of the serine racemase activity, similar to that of the full-length, membrane-bound enzyme. N-terminal sequencing of the purified His-tagged polypeptide revealed a single amino acid sequence, indicating that the formation of heterodimers between subunits of His-tagged C-VanT and endogenous alanine racemases from *E. coli* was unlikely. The authors conclude that the membrane-bound serine racemase VanT also has alanine racemase activity but is able to racemize serine more efficiently than alanine, and that the cytoplasmic domain is responsible for the racemase activity.

Keywords: D-serine, vancomycin resistance, racemases, *Enterococcus gallinarum*

INTRODUCTION

Vancomycin is a glycopeptide antibiotic that inhibits synthesis of the bacterial cell wall by binding to the acyl-D-alanyl-D-alanine (D-Ala-D-Ala) moiety of peptidoglycan precursors (Reynolds, 1989). Vancomycin resistance in *Enterococcus gallinarum* results from the synthesis of precursors ending in D-serine (D-Ser) (Reynolds

et al., 1994; Billot-Klein *et al.*, 1994a): the antibiotic has a lower affinity for UDP-MurNAc-pentapeptide[Ser] (the main peptidoglycan precursor synthesized) than for UDP-MurNAc-pentapeptide[Ala] (Billot Klein *et al.*, 1994b). Resistance requires the presence of three proteins (Arias *et al.*, 2000): VanC-1 synthesizes the dipeptide D-Ala-D-Ser (Park *et al.*, 1997) that is incorporated into peptidoglycan precursors; VanXY_C hydrolyses the dipeptide D-Ala-D-Ala and removes D-Ala from peptidoglycan precursors ending in D-Ala, ensuring the removal of 'susceptible' precursors

[†] **Present address:** Centro de Investigaciones, Universidad El Bosque, Transversal 9A no. 133-25, Santafé de Bogotá, Colombia.

(Reynolds *et al.*, 1999); and VanT converts L-serine (L-Ser) to D-Ser for peptidoglycan synthesis (Arias *et al.*, 1999).

D-Ser is not a naturally occurring amino acid, although it is produced spontaneously in the mammalian stomach due to low pH values, and it has also been reported to be a growth inhibitor of *Escherichia coli* in minimal media (McFall & Newman, 1996). Its presence has been reported in *E. coli* strains, where it serves as a readily available source of carbon (Gutnick *et al.*, 1969): a specific deaminase system converts D-Ser to pyruvate (Dowhan & Snell, 1970). Alanine racemases are pyridoxal-phosphate-dependent enzymes that catalyse the production of D-Ala from its enantiomer precursor L-Ala (Walsh, 1989). Two different alanine racemases (anabolic and catabolic) have been characterized in *Salmonella typhimurium* and *E. coli* (Wasserman *et al.*, 1983; Wild *et al.*, 1985) whereas in other bacteria investigated only one alanine racemase has been detected. The serine racemase activity of the anabolic enzyme from *S. typhimurium* involved in the synthesis of peptidoglycan was only 15% of the alanine racemase activity (Esaki & Walsh, 1986). In multicellular organisms pyridoxal-phosphate-dependent serine racemases have been characterized in the silkworm *Bombyx mori* (Uo *et al.*, 1998), where the concentration of D-Ser is increased at particular stages of metamorphosis (Corrigan & Srinivasan, 1966), and in mammalian brain (Wolosker *et al.*, 1999a), where it functions as a neuromodulator at the 'glycine site' of the N-methyl-D-aspartate receptor (Matsui *et al.*, 1995). VanT is the only serine racemase identified in bacteria so far. Unlike other alanine and serine racemases described, VanT is a transmembrane protein: at least ten transmembrane helices are predicted to be present in the N-terminal domain (Arias *et al.*, 1999) and the C-terminal domain has structural homology with the alanine racemase from *Bacillus stearothermophilus* (Shaw *et al.*, 1997), including conservation of the most important residues necessary for binding of the pyridoxal phosphate co-factor (Lys371) and for catalytic activity. Molecular modelling demonstrated that VanT could exist as a dimer (Arias *et al.*, 1999). In this paper we demonstrate that VanT exhibits significant alanine racemase activity and confirm that the C-terminal domain is sufficient for racemase activity.

METHODS

Bacterial strains and growth conditions. *E. coli* TKL-10 has a strict requirement for D-Ala to grow at 42 °C due to the presence of at least three mutations affecting alanine metabolism (Wijsman, 1972; Wasserman *et al.*, 1983). This strain and *E. coli* XL-1 Blue (Bullock *et al.*, 1987) were grown at 34 °C in Luria-Bertani (LB) broth or agar and *E. coli* M15[pREP4] (Qiagen) was grown at 37 °C in LB broth containing sorbitol (0.5 M) and betaine (2.5 mM). Ampicillin (100 µg ml⁻¹) was added for plasmid-containing derivatives of *E. coli* TKL-10 and *E. coli* XL-1 Blue and both ampicillin (100 µg ml⁻¹) and kanamycin (25 µg ml⁻¹) were added for plasmid-containing derivatives of *E. coli* M15[pREP4].

Functional complementation experiments were carried out in

E. coli TKL-10 and the same strain containing plasmid constructs expressing *vanT* (pCA4) and *alr* (pJW40). Bacteria were grown at 42 °C (non-permissive temperature) and at 30 °C simultaneously in LB agar and broth and incubated for at least 72 h.

DNA manipulations and plasmid construction. Cloning, digestion with restriction endonucleases, isolation of plasmid DNA, transformation and ligations were carried out by standard methods (Sambrook *et al.*, 1989). Plasmids pCA4 and pCA5 have been described previously (Arias *et al.*, 1999). For construction of plasmid pJW40, the *alr* gene from *B. stearothermophilus* was amplified from total DNA with primer A (5'-GATCGATCGATCCATATGAACGACTTTCATCGCGATACG-3'), which includes a *NdeI* site (underlined), and primer B (5'-GATCGAGGATCCAAGCTTT-TAGTGATGGTGATGGTGATGTGCACCTGCTTCCCGCGGCC-3'), which includes a *BamHI* site (underlined), a *HindIII* site (bold) and the sequence encoding six histidines followed by a stop codon (italics). The PCR product was cloned as a *NdeI*-*HindIII* fragment into a derivative of pUC19 (Norlander *et al.*, 1983) under the control of the *trc* promoter from pKK233-2 (Amann & Brosius, 1985).

Preparation of membrane and cytoplasmic extracts. *E. coli* TKL-10, *E. coli* XL-1 Blue and derivatives were grown in LB medium (25 ml) at 34 °C with aeration, with corresponding antibiotics if needed. When the OD₆₀₀ had reached 0.8, 0.2 mM IPTG was added and incubation continued for 2.5 h. Bacteria were harvested, washed in 150 mM Bistris propane buffer (pH 7.5), resuspended in 1 ml of the same buffer and sonicated. The broken cell preparation was centrifuged at 100 000 g for 60 min, the supernatant (cytoplasmic fraction) was collected and the pellet (membrane fraction) was washed in 150 mM Bistris propane buffer (pH 7.5) before final resuspension in 200 µl of the same buffer.

Purification of the C-terminal domain of VanT. *E. coli* M15[pREP4] (Qiagen) containing plasmid pCA5 encoding the His-tagged C-terminal domain of VanT (C-VanT) (Arias *et al.*, 1999) was grown at 37 °C in LB broth (300 ml) containing sorbitol (0.5 M) and betaine (2.5 mM) to favour the synthesis of soluble protein (Blackwell & Horgan, 1991), due to the fact that initial purification experiments indicated the presence of inclusion bodies when the gene was expressed after induction with IPTG. When the OD₆₀₀ had reached 1.0, 0.05 mM IPTG was added and incubation continued for 40 min. Bacteria were harvested at 4 °C, washed in lysis buffer (50 mM Bistris propane/300 mM NaCl/10 mM imidazole, pH 8.0) resuspended in 5 ml of the same buffer and sonicated. The broken cell preparation was centrifuged at 40 000 g for 20 min at 4 °C and the supernatant collected. All the following steps were carried out at 4 °C. The supernatant was applied to a 2 ml nickel-containing agarose column (Agarose Ni-NTA, Qiagen) equilibrated with the same buffer. The column was washed with at least 100 ml 50 mM Bistris propane/300 mM NaCl/30 mM imidazole buffer (pH 8) and His-tagged C-VanT eluted with 50 mM Bistris propane/300 mM NaCl/250 mM imidazole buffer (pH 8.0). Fractions of 0.5 ml were collected and assayed for serine racemase activity (see below).

Electrophoresis. SDS-PAGE on a 12% polyacrylamide gel was carried out under denaturing conditions using the Laemmli buffer system (Laemmli, 1970) to determine the purity and *M_r* of C-VanT. For calibration, standard proteins in the range of *M_r* 9000–175 000 (New England Biolabs) were used. Proteins were stained with 0.1% (w/v) Coomassie blue in 50% (v/v) methanol/10% (v/v) acetic acid for 30 min at 37 °C and destained with 10% (v/v) methanol/10% (v/v) acetic acid at room temperature overnight.

N-terminal sequencing. The possibility of heterodimer formation between subunits of C-VanT and endogenous alanine racemases of *E. coli* was investigated by N-terminal sequencing of the major band corresponding to the His-tagged C-VanT (M_r 43087) and a band with an M_r similar to the anabolic or catabolic alanine racemases (M_r 39128 and 38819 respectively) of *E. coli*. Proteins present in the sample containing the purified His-tagged C-VanT were precipitated with 10% (v/v) trichloroacetic acid (TCA), separated on a 12% SDS-PAGE gel and electrotransferred to a PVDF membrane. The membrane was stained with 0.1% (w/v) Coomassie blue in 50% (v/v) methanol/1% (v/v) acetic acid, destained with 50% methanol, washed in distilled water and dried. The upper and lower portions of the His-tagged C-VanT and of a band with slightly greater mobility (M_r approx. 39000) were cut out and subjected to micro-sequencing by sequential Edman degradation on a 477A sequencer in tandem with a model 120A analyser (Applied Biosystems).

Enzyme assays. Serine and alanine racemase activities of suitable dilutions of cytoplasmic and membrane fractions and of purified C-VanT were determined in a final volume of 30 μ l. The assay mixture contained 100 mM Bistris propane pH 7.5, 10 mM L-serine or L-alanine and 10 μ l of the diluted fraction as enzyme preparation. Mixtures were incubated at 37 °C for 40 min for the cytoplasmic and membrane preparations, and for 30 min at 37 °C and 42 °C for purified C-VanT. D-Amino acids produced by racemase activity were assayed using a D-amino acid oxidase assay (Messer & Reynolds, 1992), with D-serine or D-alanine as standards. The lower reliable limit of detection of D-Ser was 4 nmol min⁻¹ (mg membrane protein)⁻¹ and for D-Ala, 2 nmol min⁻¹ (mg cytoplasmic protein)⁻¹. Protein concentration was determined according to the method of Bradford (1976), with bovine serum albumin as standard.

RESULTS

Functional complementation of *E. coli* TKL-10 at 42 °C

E. coli TKL-10 is unable to grow at 42 °C in the absence of D-Ala (Wijsman, 1972). The mutations in the alanine racemases prevent the utilization of L-Ala as the source of D-Ala for peptidoglycan synthesis at the non-permissive temperature (42 °C) (Wijsman, 1972; Wassermann *et al.*, 1983). The strain did not grow at 42 °C in

the absence of an external supply of D-Ala when an enrichment medium (LB) was used (Table 1). Growth at 42 °C without a supplement of D-Ala was restored when *E. coli* TKL-10 was transformed with plasmids pCA4 or pJW40, which express *vanT* and *alr* respectively (Table 1), indicating that a basal level of expression (i.e. without induction) provided enough D-Ala for cell wall synthesis; this result also indicated that VanT was likely to possess alanine racemase activity.

Alanine racemase activities of membrane and cytoplasmic fractions

The majority (95%) of the alanine racemase activity of *E. coli* TKL-10/pJW40(*alr*) was present in the cytoplasmic fraction after induction with IPTG, whereas it was not detectable in either cytoplasmic or membrane fractions of *E. coli* TKL-10 (Table 1). The amount of alanine racemase activity in the construct carrying the *alr* gene was 150-fold greater than that present in *E. coli* XL-1 Blue/pUC18, in which the enzyme, present exclusively in the cytoplasm (Table 1) is chromosomally encoded. A similar value for the activity of the cytoplasmic alanine racemase of *E. coli* XL-1 Blue/pCA4(*vanT*) was obtained as with the strain lacking the *vanT* gene but, additionally, 24% of the total alanine racemase activity was found in the membrane fraction, presumably catalysed by VanT (Table 1). Confirmation that VanT had significant alanine racemase activity was obtained with *E. coli* TKL-10/pCA4(*vanT*): almost all (95%) the alanine racemase activity of this construct was present in the membrane fraction after induction with IPTG, a value identical to that for the distribution of serine racemase activity (Table 2).

Serine racemase activities of membrane and cytoplasmic fractions

As expected, no serine racemase activity was detected in *E. coli* TKL-10 or *E. coli* XL-1 Blue/pUC18. In the *E. coli* constructs containing the full-length *vanT* gene, the serine racemase activity was exclusively (97%) present

Table 1. Alanine racemase activities of membrane and cytoplasmic fractions of *E. coli* TKL-10 and *E. coli* XL-1 Blue and derivatives, and growth at 42 °C (non-permissive temperature for *E. coli* TKL-10)

Values represent total activities in each fraction and are the mean and standard deviation of three independent experiments with assays in duplicate. Bacteria were grown at 34 °C. ND, Not detectable.

<i>E. coli</i> strain	Alanine racemase activity (nmol min ⁻¹) in:		Growth at 42 °C
	Membrane fraction	Cytoplasmic fraction	
TKL-10	ND	ND	—
TKL-10/pJW40(<i>alr</i>)	7900 ± 990	151600 ± 9870	+
TKL-10/pCA4(<i>vanT</i>)	740 ± 125	36 ± 6	+
XL-1 Blue/pCA4(<i>vanT</i>)	336 ± 69	1020 ± 92	+
XL-1 Blue/pUC18	10 ± 1	990 ± 58	+

Table 2. Distribution of serine and alanine racemase activities between membrane (M) and cytoplasmic (C) fractions of *E. coli* TKL-10 and *E. coli* XL-1 Blue and derivatives, and activity of one enzyme as a percentage of the other activity

<i>E. coli</i> strain	Serine racemase (%) [*]		Alanine racemase (%) [*]		Ala racemase activity as % of Ser racemase activity		Ser racemase activity as % of Ala racemase activity	
	M	C	M	C	M	C	M	C
TKL-10	ND	ND	ND	ND				
TKL-10/pJW40(<i>alr</i>)	4	96	5	95	—	—	1.5	1.6
TKL-10/pCA4(<i>vanT</i>)	97	3	95	5	13.7	—	—	—
XL-1 Blue/pCA4(<i>vanT</i>)	100	0	24	76	13.7	—	—	—
XL-1 Blue/pUC18	—	—	1	99	—	—	—	0

^{*}Percentage values were calculated from the means of total activities in each fraction. ND, Not detectable.

Table 3. Serine racemase activities of membrane and cytoplasmic fractions of *E. coli* TKL-10 and *E. coli* XL-1 Blue and derivatives

Values represent total activities in each fraction and are the mean and standard deviation of three independent experiments with assays in duplicate. Bacteria were grown at 34 °C. ND, Not detectable.

<i>E. coli</i> strain	Serine racemase activity (nmol min ⁻¹) in:	
	Membrane fraction	Cytoplasmic fraction
TKL-10	ND	ND
TKL-10/pJW40(<i>alr</i>)	117 ± 10	2510 ± 32
TKL-10/pCA4(<i>vanT</i>)	5380 ± 805	142 ± 27
XL-1 Blue/pCA4(<i>vanT</i>)	2450 ± 300	ND
XL-1 Blue/pUC18	3 ± 1	ND

in the membrane fraction (Table 2). The activity of the plasmid-encoded serine racemase in both strains was substantial (two- to fivefold greater than that of the chromosomally encoded alanine racemase of *E. coli* XL-1 Blue) but less than 4% of the cytoplasmic alanine racemase of *E. coli* TKL-10/pJW40(*alr*), which was also plasmid-encoded (Table 3). The limitation in amount of VanT may have resulted from the membrane localization of the enzyme: space considerations would most likely limit the production of large amounts. The membrane-bound alanine racemase activity of both *E. coli* TKL-10 and *E. coli* XL-1 Blue carrying *vanT* on plasmids was 14% of the serine racemase activity (Table 2). The high value for alanine racemase activity in the cytoplasm of *E. coli* TKL-10/pJW40(*alr*) enabled a reliable determination to be made of the serine racemase

Table 4. Serine and alanine racemase activities of the purified C-terminus of VanT

Values represent the mean and standard deviation of three independent preparations of purified His-tagged C-terminal domain of VanT. Percentage values are in relation to serine racemase activity at 37 °C.

Temp. (°C)	Serine racemase (nmol mg ⁻¹ min ⁻¹) [%]	Alanine racemase (nmol mg ⁻¹ min ⁻¹) [%]
37	9877 ± 688 [100]	1820 ± 613 [18 ± 6.2]
42	6934 ± 812 [70 ± 8.2]	742 ± 65 [7.5 ± 0.6]

activity (Table 3) attributable to the alanine racemase of *B. stearothermophilus*, namely 1.6% (Table 2), indicating that this racemase has a much stricter specificity than VanT. The alanine racemases of *E. coli* TKL-10/pJW40(*alr*) and XL-1 Blue/pUC18 and the serine and alanine racemase activities of VanT were all inhibited by D-cycloserine: 50% inhibition occurred at between 1.4 and 2 mM cycloserine in the presence of 10 mM substrate.

Alanine and serine racemase activities of purified C-VanT

The alanine racemase activity of the purified His-tagged C-terminal domain of VanT (Fig. 1) was approximately 18% of the serine racemase activity (Table 4). Simultaneous assays of serine and alanine racemase activities performed at 37 °C and 42 °C indicated a loss of approximately 30% and 60% of the serine and alanine racemase activities, respectively, at 42 °C, in the 40 min assay (Table 4). The activities were stable at 4 °C but were lost on storage at -20 °C. The findings indicate that the C-terminal domain of VanT lacking the putative

Fig. 1. SDS-PAGE of purified His-tagged C-terminal domain of VanT (His-tagged C-VanT). Lanes: 1, sample containing His-tagged C-VanT (M_r 43 000); 2, standard proteins. The upper and lower regions of the band corresponding to His-tagged C-VanT were N-terminally sequenced. A band of M_r approximately 39 000 immediately below the band corresponding to C-VanT was also sequenced: the sequence matched the GldH (glycerol dehydrogenase) protein from *E. coli* (Truniger & Boos, 1994).

10 transmembrane segments is sufficient for serine racemase activity.

Purification of C-VanT and N-terminal sequencing

Apart from the expected M_r 43 000 band corresponding to the His-tagged C-VanT, some contaminant proteins were present after purification: bands of $M_r \geq 62 000$ and $< 32 500$ were observed on SDS-PAGE in addition to the main M_r 43 000 band (Fig. 1), although these were not considered likely to be involved in serine or alanine racemase activity. We also explored the possibility of heterodimer formation between the endogenous alanine racemase polypeptide and His-tagged C-VanT which could account for alanine racemase activity of the purified His-tagged protein. The calculated M_r of the purified His-tagged VanT is 43 087. The predicted M_r values of the endogenous anabolic and catabolic alanine racemases of *E. coli* are 39 128 and 38 819, respectively (Lobocka *et al.*, 1994; Blattner *et al.*, 1993). N-terminal sequencing was performed on both the upper and lower regions of the band corresponding to the His-tagged C-terminal domain of VanT and also on a band of slightly greater mobility (Fig. 1) (best observed when the purified protein was concentrated by TCA precipitation and

separated under denaturing conditions on a SDS-PAGE gel). The sequence corresponding to the His-tagged C-VanT was obtained from both upper and lower regions of the M_r 43 000 band (Fig. 1), and no evidence of a sequence corresponding to alanine racemases of *E. coli* was found. The sequence obtained from the band with M_r approximately 39 000 (Fig. 1) yielded the first 15 amino acids of a protein that corresponded to the glycerol dehydrogenase (GldH) from *E. coli* (M_r 38 687) (Truniger & Boos, 1994). It was concluded that the formation of heterodimers between subunits of alanine and serine racemase that could account for alanine racemase activity in the purified protein was unlikely.

DISCUSSION

VanT is an unique membrane-bound serine racemase that catalyses the synthesis of D-Ser in vancomycin-resistant *Ent. gallinarum* (Arias *et al.*, 1999). The synthesis of UDP-MurNAc-pentapeptide[D-Ser] and hydrolysis of 'susceptible' D-Ala-ending precursors is an absolute requirement for vancomycin resistance (Arias *et al.*, 2000). Serine racemases have been described in eukaryotic cells, providing D-Ser for important physiological processes. In the silk worm *Bombyx mori* D-Ser may play an important role in development and metamorphosis (Corrigan & Srinivasan, 1966). In mammalian brains D-Ser functions as an important neuromodulator (Wolosker *et al.*, 1999a). Neither of these two enzymes appears to have a membrane association (Wolosker *et al.*, 1999a; Uo *et al.*, 1998). In *E. coli* D-Ser can act as a sole source of carbon and nitrogen (McFall & Newman, 1996). However, D-Ser seems to be toxic for *E. coli*, inhibiting enzymes for panthothenate and L-serine synthesis (Cosloy & McFall, 1973). Moreover, *E. coli* has a specific deaminase enzyme system for the catabolism of D-Ser (Dowhan & Snell, 1970). It has been proposed that the natural source of D-Ser in *E. coli* is the non-enzymic racemization of L-Ser (Friedman, 1991). It is clear that vancomycin-resistant *Ent. gallinarum* has developed a specific enzyme to provide D-Ser for peptidoglycan synthesis. The C-terminal domain of VanT has substantial sequence identity with alanine racemases of different organisms (Arias *et al.*, 1999), especially with that of *B. stearothermophilus*, whose crystal structure has been solved (Shaw *et al.*, 1997). Biochemical evidence presented here indicates that the C-terminal domain of VanT also catalyses the racemization of L-Ala. Alanine racemase activity was evident *in vivo*, when a plasmid expressing *vanT* was able to complement *E. coli* TKL-10 at the non-permissive temperature (42 °C). Interestingly, we did not detect any alanine racemase activity in *E. coli* TKL-10 even at the permissive temperature (30 °C), a finding which is in agreement with the initial description of this strain by Wijsman (1972). Although the absence of enzyme activity at the permissive temperature has also been described in other temperature-sensitive mutants (Eidlic & Neidhardt, 1965), it is likely that the sensitivity of the alanine racemase assay used here was insufficient to detect a basal, low activity of the enzyme, as has been

Fig. 2. Alignment (CLUSTAL W; Thompson *et al.*, 1994) of the C-terminal domain of VanT (GenBank accession no. AF 077816) with the anabolic (Alr1) and catabolic (Alr2) alanine racemases from *E. coli* (Wild *et al.*, 1985). Black boxes indicate amino acid identity. Shaded boxes indicate similar amino acids. The putative pyridoxal phosphate attachment site of VanT, Alr1 and Alr2 is shown above the alignment. The predicted N-terminal β/α , and the C-terminal β subdomains of VanT (Arias *et al.*, 1999) include amino acids 351–558 and 575–698 respectively.

shown before in other strains of *E. coli* when the level of alanine racemase is below 4% of the normal activity (Wassermann *et al.*, 1983). The appearance of such activity in the membrane fraction when the strain was transformed with pCA4 further supports the finding that full-length VanT also catalyses racemization of L-Ala *in vivo*.

The alanine racemase activity of the purified His-tagged C-VanT was approximately 18% relative to its serine racemase activity (Table 4), similar to that of the membrane-bound enzyme (Table 2). Alanine racemase activities of the serine racemases of brain and silkworms are considerably less (1.5% and 6% of the serine racemase activity respectively) (Wolosker *et al.*, 1999a; Uo *et al.*, 1998), indicating that the cytoplasmic domain of VanT is less selective than those of the eukaryotic enzymes. Recently, Wolosker *et al.* (1999b) reported the cloning and sequencing of the pyridoxal-phosphate-dependent serine racemase from rat brain. Although the protein sequence does not exhibit significant homology

with VanT it is likely that the mechanism of racemization of L-Ser is conserved in both proteins.

As VanT is likely to exist as a dimer (Arias *et al.*, 1999) we explored the possibility of heterodimer formation between polypeptides of alanine racemase from *E. coli* and VanT. Cross-species heterodimers have been reported in different enzymes (Osterman *et al.*, 1994; Sun *et al.*, 1992; Greene *et al.*, 1993). Active cross-species heterodimers are usually not formed if the subunits share less than 80% identity in the dimer interface (Osterman *et al.*, 1994). An alignment between VanT and the alanine racemases of *E. coli* (Fig. 2) revealed that the overall sequence identity is only 33%. Based on the crystal structure of Alr from *B. stearothermophilus* (Shaw *et al.*, 1997) we previously predicted that the residues from the other subunit of VanT involved in binding or catalysis could be Tyr597, Asp647 and Met648 (replaced by Gln314 in Alr) (Arias *et al.*, 1999), which are also conserved in the alanine racemases from *E. coli* (Fig. 2). However, it is unlikely that the sequence

identity between the subunits of these proteins at the dimer interface is as high as 80%. Also we did not find evidence of heterodimer formation based on N-terminal sequencing of different parts of the band of the purified His-tagged C-VanT from the SDS-PAGE gel or from sequencing of a protein with a similar M_r to that of alanine racemase that was eluted with the purified His-tagged C-VanT from the nickel column (Fig. 1). The sequence obtained did not correspond to alanine racemases (instead it matched a glycerol dehydrogenase). One possible explanation for the lack of detection of subunits of alanine racemases is that being in a small proportion compared to the His-tagged C-VanT, they undergo blocking at the N-terminus and therefore become unavailable for Edman degradation. These results indicate that formation *in vivo* of heterodimers between subunits of alanine racemases of *E. coli* and the C-terminal domain of VanT is unlikely but if such heterodimers are present their proportion is likely to be very small compared to the formation of homodimers of C-VanT. Another possibility is that the topological organization of the transmembrane and cytoplasmic domains of VanT may play a role in amino acid selectivity for racemization. Investigation of this interaction is currently in progress.

In summary, VanT is a membrane-bound serine racemase that also exhibits alanine racemase activity. The C-terminal domain of VanT is sufficient for catalysis and also has both activities.

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