The Physiological Contribution of *Acinetobacter* PcaK, a Transport System That Acts upon Protocatechuate, Can Be Masked by the Overlapping Specificity of VanK†

DAVID A. D'ARGENIO, ANA SEGURA,‡ WAYNE M. COCO,§ PATRICIA V. BÜNZ,||
AND L. NICHOLAS ORNSTON*

Department of Molecular, Cellular and Developmental Biology, Yale University,
New Haven, Connecticut 06520-8103

Received 21 December 1998/Accepted 30 March 1999

VanK is the fourth member of the ubiquitous major facilitator superfamily of transport proteins to be identified that, together with PcaK, BenK, and MucK, contributes to aromatic catabolism in *Acinetobacter* sp. strain ADP1. VanK and PcaK have overlapping specificity for *p*-hydroxybenzoate and, most clearly, for protocatechuate: inactivation of both proteins severely impairs growth with protocatechuate, and the activity of either protein alone can mask the phenotype associated with inactivation of its homolog. Furthermore, *vanK* *pcaK* double-knockout mutants appear completely unable to grow in liquid culture with the hydroaromatic compound quinate, although such cells on plates convert quinate to protocatechuate, which then accumulates extracellularly and is readily visible as purple staining. This provides genetic evidence that quinate is converted to protocatechuate in the periplasm and is in line with the early argument that quinate catabolism should be physically separated from aromatic amino acid biosynthesis in the cytoplasm so as to avoid potential competition for intermediates common to both pathways. Previous studies of aromatic catabolism in *Acinetobacter* have taken advantage of the ability to select directly strains that contain a spontaneous mutation blocking the β -ketoadipate pathway and preventing the toxic accumulation of carboxymuconate. By using this procedure, strains with a mutation in structural or regulatory genes blocking degradation of vanillate, *p*-hydroxybenzoate, or protocatechuate were selected. In this study, the overlapping specificity of the VanK and PcaK permeases was exploited to directly select strains with a mutation in either *vanK* or *pcaK*. Spontaneous mutations identified in *vanK* include a hot spot for frameshift mutation due to contraction of a G₆ mononucleotide repeat as well as point mutations producing amino acid substitutions useful for analysis of VanK structure and function. Preliminary second-site suppression analysis using transformation-facilitated PCR mutagenesis in one VanK mutant gave results similar to those using LacY, the prototypic member of the major facilitator superfamily, consistent with the two proteins having a similar mechanism of action. The selection for transport mutants described here for *Acinetobacter* may also be applicable to *Pseudomonas putida*, where the PcaK permease has an additional role in chemotaxis.

One of the defining characteristics of a microorganism is the spectrum of compounds that are transported across the cell membrane, and it is therefore of interest to examine the diversity of proteins that have evolved to contribute to an organism's transport capacity (7). Genome sequencing has revealed that a significant fraction of transport proteins (64) can be grouped either into the ATP-binding cassette (ABC) superfamily or the major facilitator superfamily. In the soil bacterium *Acinetobacter* sp. strain ADP1, permeases associated with the β -ketoadipate pathway for catabolism of aromatic compounds appear exclusively to be members of the major facilitator superfamily (61): BenK (9) and MucK (77) are involved in uptake of compounds in the benzoate branch of the path-

way, and PcaK (8, 45) is involved in the *p*-hydroxybenzoate branch. This study concerns VanK, a fourth member of the superfamily associated with aromatic catabolism in *Acinetobacter*.

Studies of the β -ketoadipate pathway in *Acinetobacter* have taken advantage of the ability to use a strain with the engineered Δ *pcaBDK1* deletion to directly select derivatives in which a spontaneous mutation prevents the toxic accumulation of carboxymuconate (Fig. 1). By using this procedure, strains that contained a structural gene mutation inactivating the VanAB vanillate demethylase (69), the PobA *p*-hydroxybenzoate hydroxylase (13, 33), or the PcaHG protocatechuate dioxygenase (12, 28) were selected. Also selected were strains with a regulatory gene mutation inactivating either of two transcriptional activators (14, 26): PobR (14, 27, 43, 44), governing expression of PobA, or PcaU (11), governing expression of PcaH and PcaG (31). As described in this study, the gene for the PcaK transport protein is already inactivated by the Δ *pcaBDK1* deletion in the parental strain ADP500, which consequently allowed the selection of derivatives with a mutation in VanK, a protein closely related to and overlapping in specificity with PcaK.

The genetic analysis presented here was made possible by characterization of a chromosomal segment containing *vanA* and *vanB*, structural genes for vanillate demethylase (69). As

* Corresponding author. Mailing address: Department of Molecular, Cellular and Developmental Biology, Yale University, P.O. Box 208103, New Haven, CT 06520-8103. Phone: (203) 432-3498. Fax: (203) 432-3497. E-mail: nicholas.ornston@yale.edu.

† Publication 20 from the Biological Transformation Center in the Yale Biospherics Institute.

‡ Present address: Department of Biochemistry, Consejo Superior de Investigaciones Científicas, Estación Experimental de Zaidín, 18012 Granada, Spain.

§ Present address: Energy Biosystems, The Woodlands, Tex.

|| Present address: Institut für Allgemeine Botanik, Abteilung Mikrobiologie, Hamburg, Germany.

FIG. 1. Positive selection of mutants blocked in catabolism of aromatic or hydroaromatic compounds. Strain ADP500 contains the engineered Δ pcaBDK1 deletion and cannot grow with succinate in the presence of protocatechuate (or compounds upstream in the β -ketoadipate pathway) due to the toxic accumulation of β -carboxy-*cis,cis*-muconate. ADP500 derivatives in which a spontaneous mutation blocks the β -ketoadipate pathway upstream of carboxymuconate can therefore be selected. After correction of the Δ pcaBDK1 deletion to wild type, the phenotype of the remaining spontaneous mutation can be revealed by growth tests with single carbon sources.

shown in Fig. 2, these genes, essential for growth with vanillate, neighbor an open reading frame designated *vanK* on the basis of its genetic location and its sequence similarity to other transporters associated with aromatic catabolism (69). The other investigation (69) did not associate a phenotype with *vanK*. That knowledge emerged from the present study, initiated by discovery that in strains defective in *pcaK*, mutations in *vanK* protect cells against toxicity exerted by protocatechuate. The *vanK* mutations occur with remarkable frequency, and so double mutants unable to express both protocatechuate oxygenase and VanK are likely to emerge from exposure of cells to protocatechuate in a single round of mutant selection (Fig. 1). The genetic bases for the genetic instability of *vanK* are reported here and elsewhere (69). Mutations blocking both *pcaK* and *vanK* impede growth with either protocatechuate or quinate (Fig. 1) and cause extracellular accumulation of protocatechuate from the latter compound. *vanK* is the first observed chromosomal locus that can specifically influence protocatechuate catabolism and is not within the *pca-qui-pob* supraoperonic cluster (17, 30, 35).

FIG. 2. Chromosomal region containing *vanA* and *vanB*, genes for vanillate demethylase. Arrows denote direction of transcription. DNA fragments cloned in plasmids pZR139, pZR143, and pZR144 (69) were used to map *vanK* mutations by marker rescue.

MATERIALS AND METHODS

Strains and culture conditions. *Acinetobacter* sp. strain ADP1, originally called *Acinetobacter calcoaceticus* BD413 (39), was routinely grown with 10 mM succinate in a mineral medium. Unless otherwise indicated, cells were grown at 37°C and the mineral medium was supplemented with 5 mM *p*-hydroxybenzoate, 3 mM quinate, 3 mM vanillate, 3 mM protocatechuate, or 2.5 mM benzoate. Because of the instability of protocatechuate, only fresh plates or liquid cultures with this carbon source were used, made with stock solutions (pH 7.0) stored frozen until use. For characterization of mutant phenotypes, strains were tested after overnight growth on plates with 10 mM succinate.

DNA manipulations. Crude cell lysates of *Acinetobacter* strains for use in transformation reactions were prepared by incubation of pelleted cells (from a 5-ml culture) in 0.5 ml of saline-citrate buffer with 0.05% sodium dodecyl sulfate at 60°C for 1 h. Plasmids were isolated from 5-ml *Escherichia coli* cultures by using the Wizard miniprep kit (Promega) and assumed to contain 2 μ g of DNA. To isolate template DNA for PCR, pelleted *Acinetobacter* cells from a 5-ml culture were washed and treated with InstaGene Matrix as described by the supplier (Bio-Rad); 5 μ l of the resulting solution was then used in 50- μ l PCRs including 0.5 U of *Taq* polymerase (Boehringer Mannheim), 10 pmol of each primer, and 10 pmol of each deoxynucleoside triphosphate. Standard PCR conditions were used: 30 cycles of denaturing at 94°C for 45 s, annealing at 56°C for 45 s, and elongation at 72°C for 1 min 30 s. PCR primers were synthesized by the Keck Biotechnology Resource Laboratory (Yale University).

For efficient transformation of *Acinetobacter* strains, 200 μ l of a 5-ml culture of the recipient strain grown overnight with 10 mM succinate was transferred to a fresh 5-ml culture. After growth in a gyratory shaker for 2 h (to increase competence), 500 μ l of the culture was transferred to Falcon 15-ml polypropylene tubes together with 1 μ g of plasmid DNA or 20 μ l of a 50- μ l PCR mixture. After overnight shaking incubation, transformation reactions were transferred to appropriate selective plates.

Selection and characterization of strains deficient in *vanK*. Strain ADP500 (32) contains the engineered Δ catD101::Km^r and Δ pcaBDK1 mutations. To isolate mutants blocked in catabolism of protocatechuate, single colonies of succinate-grown ADP500 were transferred to patches on freshly prepared plates with 10 mM succinate and 3 mM protocatechuate. Cells with spontaneous secondary mutations blocking protocatechuate catabolism do not accumulate toxic levels of β -carboxy *cis,cis*-muconate and are able to grow (Fig. 1). To prevent analysis of siblings, only one mutant derivative was picked per single colony of ADP500.

To demonstrate coselection of independent mutations in *pcaH* and *pcaG* and in *vanK*, mutant cells of ADP500 were selected for resistance to 3 mM protocatechuate at 37°C and then screened for heat-sensitive mutations in *pcaH* and *-G*. Correction of the Δ pcaBDK1 deletion in 16 such strains generated ADP1102 to ADP1117, each with a spontaneous heat-sensitive mutation in either *pcaH* or *-G* (12). To identify and to map potential spontaneous mutations in *vanK* in addition to the conditional mutation in *pcaH* or *-G*, the Δ pcaBDK1 deletion in the 16 strains was replaced on the chromosome with Δ pcaK859 (8) instead of wild-type DNA by the following procedures. Recipient strains were patched onto an LB plate together with 1 μ l of crude cell lysate of ADP859 (Δ pcaK859). After overnight growth, cells from each patch were transferred to and purified on plates with 5 mM *p*-hydroxybenzoate incubated at 37°C, thereby demanding correction of the heat-sensitive mutation in *pcaH* or *-G* as well as activity of PcaBD. This generated strains ADP7576 to ADP7591 (Table 1; equivalent to strains ADP1102 to ADP1117 with the addition of Δ pcaK859), which were then tested for growth with quinate. Strains severely impaired in growth with quinate (or protocatechuate) due to inactivation of both *vanK* and *pcaK* were patched onto an LB plate together with about 50 ng of plasmid pZR139, pZR143, or pZR144 (69) (Fig. 2). After overnight growth, cells from each patch were transferred to a plate with 3 mM quinate: wild-type growth with quinate indicated marker rescue and allowed rough localization of the spontaneous *vanK* mutation.

An independent collection of mutants was obtained by selecting derivatives of ADP500 mutated so that they resisted 3 mM protocatechuate at 22°C. Strains from this selection (still containing the Δ pcaBDK1 deletion) with a cold-enhanced resistance to *p*-hydroxybenzoate were found to have a spontaneous mutation affecting transcriptional regulation by PcaU and are described elsewhere. Strains that resisted protocatechuate but not *p*-hydroxybenzoate at both 37 and 22°C were purified on an LB plate and then tested for the ability to grow on a plate with 10 mM succinate in the presence of 3 mM vanillate (which does not require a functional *vanK* for its metabolism). Strains from this selection (still containing the Δ pcaBDK1 deletion) with a cold-enhanced resistance to *p*-hydroxybenzoate were found to have a spontaneous mutation affecting transcriptional regulation by PcaU and were described elsewhere (11). Strains not resistant to *p*-hydroxybenzoate at either 37 or 22°C were purified on an LB plate and then tested for the ability to grow on a plate with 10 mM succinate in the presence of 3 mM vanillate. For those strains also not resistant to vanillate and therefore assumed to have a spontaneous mutation confined to *vanK*, crude cell lysates were made. To identify complementation groups, these lysates were tested for the ability to transform strains carrying Δ pcaK859 combined with various sequenced mutations in *vanK* to wild-type growth with quinate: 5-ml cultures of the double-mutant recipient strains were grown overnight with 10 mM succinate and, after addition of 10 μ l of 1 M succinate, incubated for an additional 30 min in a gyratory shaker (to increase competence) before being spread

TABLE 1. Strains mutated in *vanK*

Strain ^a	Genotype		<i>vanK</i> mutation	VanK mutation
	<i>pca</i>	<i>van</i>		
ADP7576	Δ <i>pcaK859</i>	Δ <i>van1102</i>		
ADP7577 ^b	Δ <i>pcaK859</i>	Δ <i>vanK1103</i>	Δ G (366–371) ^d	
ADP7579	Δ <i>pcaK859</i>	Δ <i>van1105</i>		
ADP7580	Δ <i>pcaK859</i>	Δ <i>van1106</i>		
ADP7582	Δ <i>pcaK859</i>	Δ <i>vanK1108</i>	Δ A (697–704) ^d	
ADP7584	Δ <i>pcaK859</i>	Δ <i>van1110</i>		
ADP7587	Δ <i>pcaK859</i>	Δ <i>vanK1113</i>	G212A	Gly71Asp
ADP7592	Δ <i>pcaBDK1</i>	Δ <i>vanK1103</i>	Δ G (366–371) ^d	
ADP7593	Δ <i>pcaBD1</i>	Δ <i>vanK1103</i>	Δ G (366–371) ^d	
ADP7618		Δ <i>vanK1103</i>	Δ G (366–371) ^d	
ADP7602 ^c	Δ <i>pcaK859</i>	Δ <i>vanK7602</i>	Δ (A427–T522)	Δ (Ser143–Arg174)
ADP7603	Δ <i>pcaK859</i>	Δ <i>vanK7603</i>	<i>vanK</i> ::IS1236	
ADP7604	Δ <i>pcaK859</i>	Δ <i>vanK7604</i>	G460T	Gly154Trp
ADP7607	Δ <i>pcaK859</i>	Δ <i>vanK7607</i>	Δ (T769–A918)	Δ (Cys257–Ala306)
ADP7608	Δ <i>pcaK859</i>	Δ <i>vanK7608</i>	C655T	Gln219Stop
ADP7609	Δ <i>pcaK859</i>	Δ <i>vanK7609</i>	G1133C	Arg378Pro
ADP7610	Δ <i>pcaK859</i>	Δ <i>vanK1113</i>	G212A	Gly71Asp
		Δ <i>vanK7610</i>	T806C	Phe269Ser
		Δ <i>vanK7611</i>	T812C	Phe271Ser
ADP7612	Δ <i>pcaK859</i>	Δ <i>vanK1113</i>	G212A	Gly71Asp
		Δ <i>vanK7612</i>	G880C	Gly294Arg

^a ADP7576 to ADP7591 are derived from strains selected for a spontaneous mutation in *pcaH* or *pcaG*; ADP7602 to ADP7609 are derived from strains selected for a spontaneous mutation solely in *vanK*; ADP7610 and ADP7612 are derived from ADP7587 and contain PCR-generated suppressor mutations of *vanK1113*.

^b Same as ADP7578, ADP7581, ADP7583, ADP7585, ADP7586, ADP7589, and ADP7590.

^c Same as ADP7605, and ADP7606.

^d Frameshift mutation caused by deletion of one residue in a mononucleotide repeat.

onto selective plates with 3 mM quinate. Lysate (4 μ l) of each mutant was spotted onto the cell lawn. Because the Δ *pcaBDK1* deletion in the donor strains overlapped the Δ *pcaK859* mutation in the recipients, wild-type growth with quinate was possible only when the lysate DNA corrected the *vanK* mutation; no growth was readily observed when the donor and recipient contained the same or overlapping mutations in *vanK*. Strains which appeared to have a unique *vanK* mutation were treated as described above: Δ *pcaBDK1* was replaced on the chromosome with Δ *pcaK859*, generating ADP7602 to ADP7609 (Table 1), and the *vanK* mutation was mapped by marker rescue.

To reconstruct and to analyze the selection pressure for *vanK* inactivation, the Δ *pcaBDK1* and Δ *pcaBD1* deletions each were introduced into the chromosome of an ADP500 derivative carrying only the spontaneous *vanK1103* mutation, generating ADP7592 and ADP7593, respectively (Table 1). To construct such strains, liquid cultures were transformed (as described above) with one of two plasmids: pZR301 (32), in which removal of two internal *EcoRV* fragments from the pZR3 (28, 32) insert generated Δ *pcaBDK1*; and pZR35, in which removal of one of the internal *EcoRV* fragments generated Δ *pcaBD1*. Transformation reactions were streaked onto plates with 10 mM succinate, and single colonies were tested for loss of the ability to grow with *p*-hydroxybenzoate, indicating chromosomal replacement of wild-type *pca* genes with the engineered deletion. Generally at least 1 in 500 colonies tested had the desired phenotype.

Transformation-facilitated PCR mutagenesis. For mutagenesis (12, 43, 44), standard PCR was performed as described above except that the number of cycles was increased to 35. For suppression of *vanK1113*, a 1.2-kb PCR fragment was amplified from ADP7587 by using primers VK3 (5'-GTTTAGCTATTGCA GGATCAGC-3') and VK6 (5'-GCAATAAATTCGGCATGGACTAC-3'). For mutagenesis of *pcaK*, a 1.7-kb fragment was amplified from ADP7593 by using primers PK1 (5'-CATTGATTATCGCGGGTAGTGC-3') and PK2 (5'-CTCAAGTGAACGGTTAATCATGC-3'). Twenty microliters of each PCR mixture was directly used to transform a liquid culture of the recipient strain (as described above).

DNA sequence analysis. To isolate template DNA for sequencing, products of PCRs performed under standard conditions were purified with 8 μ l of Gene-Clean Glassmilk according to the supplier (Bio 101, Inc.) and resuspended in 25 μ l of water; 8 μ l was used for ABI PRISM dye terminator cycle sequencing with AmpliTaq DNA polymerase, FS (Perkin-Elmer). Cycle sequence reactions were processed as previously described (43). PCR primers were synthesized by the Keck Biotechnology Resource Laboratory (Yale University), which also performed some of the DNA sequencing. The nucleotide sequence of a 14-kb *EcoRI*

fragment containing *Acinetobacter* wild-type *vanK* has been reported previously (69) and appears in the GenBank nucleotide sequence database under accession no. AF009672.

RESULTS

Additional mutations blocking conversion of vanillate to protocatechuate in strains selected directly for a block in protocatechuate utilization. Selection for resistance to the toxic accumulation of carboxymuconate in the engineered *Acinetobacter* mutant ADP500 yields strains with secondary mutations blocking the β -ketoadipate pathway upstream of PcaB (Fig. 1). By using this procedure, a collection of strains each with a spontaneous mutation in *pcaH* or *pcaG*, structural genes for protocatechuate 3,4-dioxygenase (12, 28, 31), was generated. One such mutant, ADP6311, contained a 302-bp deletion in *pcaG* (28). As part of a study of catabolic pathways in *Acinetobacter* potentially convergent upon protocatechuate, it was shown that unlike the wild-type strain ADP1, ADP6311 and other mutants blocked in *pcaH* and *-G* could not grow with vanillate as sole carbon and energy source. This was expected since the first step in vanillate catabolism is its conversion to protocatechuate by vanillate demethylase (69) (Fig. 1). However, when the supernatant of an ADP6311 culture grown in the presence of vanillate was analyzed by high-pressure liquid chromatography (data not shown), no accumulation of protocatechuate was detected, indicating that contrary to expectation, the vanillate remained unmetabolized by this strain. Furthermore, ADP6338 (28), a spontaneous mutant containing a leaky four amino acid deletion in PcaH causing slow growth with protocatechuate, was unable to grow with vanillate.

Since ADP6311 and ADP6338 came from the same collection of spontaneous mutants, the anomalous defect in vanillate catabolism could have been the result of a mutation in the particular isolate of the parental strain ADP500. Therefore, we tested a separate collection of 16 strains (ADP1102 to ADP1117) derived from ADP500 and each with a heat-sensitive mutation in *pcaH* or *-G* (12) for growth with vanillate at the permissive temperature (22°C). Although all 16 strains grew with *p*-hydroxybenzoate at 22°C, four (ADP1102, ADP1105, ADP1106, and ADP1110) could not grow at 22°C with vanillate (Fig. 1). This finding confirmed the original observation with ADP6311 and ADP6338 and indicated that strains selected for a block in conversion of protocatechuate to carboxymuconate could contain a significant fraction with an additional independently selected block in conversion of vanillate to protocatechuate. Suggesting that this additional spontaneous mutation in ADP6338 was a large deletion, transformation of ADP6338 with chromosomal DNA contained in crude cell lysate of wild-type strain ADP1 yielded only rare recombinants able to grow with vanillate.

Identification of *vanK*. The apparent presence of a deletion mutation preventing vanillate degradation in ADP6338 raised the possibility that a selected mutation blocked *vanK* (which proved not to be required for vanillate degradation) and that the deletion mutation extended into *vanA* and *vanB* (which are required for vanillate degradation [69] [Fig. 2]). Therefore, other ADP500-derived strains with no apparent block in vanillate degradation may also harbor an undetected mutation, and ADP1103 (12) was used to test this possibility. By demanding growth with *p*-hydroxybenzoate at 37°C, a strain in which the heat-sensitive *pcaH1103* mutation had spontaneously reverted was selected, and a subsequent test showed that growth with vanillate was not detectably impaired. To reveal any mutations other than *pcaH1103* that might have been originally selected in this strain, the Δ *pcaBDK1* mutation was reintroduced into

FIG. 3. Nucleotide sequence of *vanK* and deduced amino acid sequence of the encoded protein. Underlined with a dashed line are 12 segments with amino acids potentially forming transmembrane α -helices (identified by the TMpred program of the ISREC-Bioinformatics Group, Geneva, Switzerland). The orientation in the membrane of these segments is predicted to alternate with the first helix oriented inside to outside. Sequenced spontaneous mutations are indicated above *vanK* nucleotides and above *VanK* amino acids; all were selected based on loss of *VanK* function except for the intragenic suppressors of *vanK1113*: the underlined *vanK7610* and *vanK7611* (together in ADP7610) and *vanK7612* (in ADP7612). The TAG sequence duplicated as a result of IS1236 insertion in *vanK7603* is underlined below a vertical arrow. Underlined below an open triangle are the G₆ and A₇ mononucleotide repeats in which 1 bp is deleted, in Δ*vanK1103* and Δ*vanK1108*, respectively. Horizontal arrows are shown above the first and last nucleotides deleted in Δ*vanK7607* and Δ*vanK7602*, the latter deletion including one of two repeats of the 8-bp sequence GCTGGCGT (underlined). Downstream from *vanK* is a gene for a putative porin (69) (Fig. 2).

the chromosome (see Materials and Methods), generating ADP7592 (Table 1). Reconstructing the selection for resistance to carboxymuconate accumulation, it was found that ADP7592, unlike the parental strain ADP500, appeared completely resistant to 1 mM protocatechuate but the 3 mM concentration used in the original selection remained toxic.

The phenotype of ADP7592, sensitive to the difference between 1 and 3 mM protocatechuate, suggested the presence of a hitherto undetected spontaneous *vanK* mutation affecting protocatechuate catabolism at the level of transport. Fortunately coincident with this study, a genetic region including genes for conversion of vanillate to protocatechuate was cloned from *Acinetobacter* (69). DNA sequencing of this region revealed *vanA* and *vanB*, encoding the two subunits of vanillate demethylase, and, transcribed divergently from these structural genes, a gene encoding a protein closely related to the *Acinetobacter* (8, 45) and *Pseudomonas* (34, 56) PcaK permease (Fig. 2). This gene was designated *vanK* to reflect its chromosomal location but the role of *vanK* in vanillate catabolism remains uncertain (see Discussion). Preliminary studies in

Acinetobacter (8) indicate that PcaK is involved in uptake of *p*-hydroxybenzoate and protocatechuate as is the case in *Pseudomonas* (34, 56). Given the sequence similarity of *VanK* and PcaK, the *vanK* gene seemed an ideal candidate for a target of selection that could affect resistance of ADP500 to protocatechuate.

Nucleotide sequence of *vanK*. The *vanK* gene (Fig. 3) encodes a protein of 448 amino acids ($M_r = 47,927$) whose sequence indicates that it is a member of the major facilitator superfamily of transport proteins (52, 61). In a phylogenetic analysis (Fig. 4), *VanK* clusters with six other proteins: PcaK (45) and BenK (9) in *Acinetobacter* strain ADP1, PcaK (34, 56) in *Pseudomonas putida*, MhpT (20) in *E. coli*, TfdK (48) in *Ralstonia eutropha*, and HppK (2) in *Rhodococcus globerulus*. These six permeases, known or predicted to mediate the uptake of aromatic acids, form a new protein family within the superfamily (2, 61). *VanK* is most closely related (37% identity over 442 amino acid aligned residues [1]) to HppK, predicted to transport 3-hydroxyphenylpropionate, also the predicted substrate of MhpT. As with LacY, the prototypic member of

FIG. 4. Relative sequence divergence in the aromatic acid permease family. The organisms in which these proteins were identified and the substrates, inferred or demonstrated, for the transporters are as follows: PcaK (Ac) in *Acinetobacter* sp. strain ADP1, protocatechuate and *p*-hydroxybenzoate (reference 45 and this study); PcaK (Ps) in *P. putida*, protocatechuate and *p*-hydroxybenzoate (34, 56); MhtP in *E. coli*, 3-(3-hydroxyphenyl)propionate (20); BenK in *Acinetobacter* sp. strain ADP1, benzoate (9); TfdK in *Ralstonia eutropha*: 2,4-dichlorophenoxyacetate (48); HppK in *Rhodococcus globerulus*: 3-(3-hydroxyphenyl)propionate (2); VanK in *Acinetobacter* sp. strain ADP1, protocatechuate and *p*-hydroxybenzoate (this study); MucK in *Acinetobacter* sp. strain ADP1, *cis,cis*-muconate (77). The degree to which each of these proteins may facilitate transport of multiple related compounds remains unknown. A new family of carboxylic acid permeases including MucK, distinct from the aromatic acid permease family (61), may become apparent with additional sequencing (2). The sequence of *Acinetobacter* PcaK in GenBank has been updated (accession no. AF009672, June 1998).

the major facilitator superfamily, VanK and the other members of the aromatic acid permease family are predicted to contain 12 membrane-spanning α -helical segments (Fig. 3), topologically oriented so that a large cytoplasmic loop is formed by residues in the middle of the linear amino acid sequence. An amino acid signature sequence for this family (61), including most of the residues in transmembrane segment 2, is matched in 11 of 13 positions in the VanK sequence (Fig. 3, VanK residues 62 to 81). This signature sequence in turn just overlaps with amino acids between transmembrane segments 2 and 3 that had been previously identified as characteristic of the superfamily (51, 61).

Spontaneous mutations inactivating *vanK* can be masked by PcaK activity. The Δ *pcaBDK1* mutation in ADP500, besides blocking the β -ketoadipate pathway at the level of carboxymuconate, also inactivates *pcaK*. This loss of *pcaK* function, however, does not protect ADP500 from the toxic accumulation of carboxymuconate during growth in the presence of even micromolar quantities of protocatechuate. Furthermore, growth on plates with protocatechuate is not dramatically impeded in ADP859 (8), in which the Δ *pcaK859*-engineered 4-bp deletion (of one of the tandem TTAA repeats between nucleotides [nt] 4945 and 4952 [45]) inactivates the encoded permease in an otherwise wild-type genetic background. This apparent redundancy of *pcaK* function suggested that spontaneous mutations in *vanK* might contribute to protocatechuate resistance in ADP500 but then go undetected after PcaK activity was restored by correction of *pcaBDK* to wild type.

To reveal *vanK* mutations potentially masked by PcaK activity in the 16 spontaneous mutant strains, ADP1102 to ADP1117 (12), the heat-sensitive mutation in *pcaH* or *pcaG* in each strain was corrected to wild type, and simultaneously the Δ *pcaK859* mutation was introduced into the chromosome, inactivating *pcaK* (see Materials and Methods). Tests of the resulting strains, ADP7576 to ADP7591 (Table 1), revealed that 14 of 16, including the four strains with apparent deletions overlapping *vanK*, were now severely impaired in growth with

protocatechuate or quinate. Excluding the four deletion mutants, the spontaneous mutation in the remaining 10 strains was localized to *vanK* by marker rescue with cloned DNA in plasmids pZR139, pZR143, and pZR144 (69) (Fig. 2). Subsequently, DNA sequencing of the appropriate region, in 8 of the 10 strains, revealed Δ *vanK1103* (Fig. 3), a single-base deletion in a string of six G residues (nt 366 to 371). The remaining two strains had different mutations (Fig. 3): Δ *vanK1108*, a single-base deletion in a string of seven A residues (nt 697 to 704) or *vanK1113*, a point mutation causing substitution of Gly₇₁ with Asp₇₁ in the VanK protein. These results confirm the prediction that *vanK* can be a target of spontaneous mutation during selection with strain ADP500 and that these mutations can be masked by the activity of PcaK.

Role of *vanK* during selection of ADP500 derivatives resistant to protocatechuate. The reconstruction experiment with ADP7592 showed that the Δ *vanK1103* spontaneous mutation alone could not provide resistance to protocatechuate at the concentration (3 mM) used in the original selection. This explains the necessity for the *pcaH1103* mutation in addition to Δ *vanK1103* in the original ADP500 derivative but does not explain the order in which these two mutations arose. To determine when *vanK* mutations may have arisen, the selection for mutant derivatives of ADP500 was repeated. Mutant colonies growing in the presence of 3 mM protocatechuate were purified by two successive transfers on selective plates as in the original selections (12, 28). Mutant cells generally grew poorly during these transfers, and frequently there appeared a mix of colony sizes. In a particularly clear case after the second round of transfer, a crude cell lysate was made from one relatively large colony. In contrast to lysates of other samples of this mutant strain from the first and second transfers, the DNA in the lysate of the large colony could not transform ADP7576 (Table 1), a strain deleted in *vanK* and containing the Δ *pcaK859* mutation, to wild-type growth with protocatechuate. This negative transformation result indicates that the large colony had acquired an additional mutation preventing correction of the ADP7576 *vanK* defect and implies that a mutation inactivating *vanK* can arise by unintended selection during purification on selective media of a mutant strain already impaired in protocatechuate degradation.

Direct selection and characterization of strains with a spontaneous mutation inactivating *vanK*. The resistance of ADP7592 (Δ *vanK1103* Δ *pcaBDK1*) to growth at 37°C in the presence of 1 mM or less protocatechuate suggested that these conditions could be used to directly select ADP500 derivatives with spontaneous mutations only in *vanK*. Indeed, such strains had almost certainly already been isolated: it had been previously noted (65) that by using these conditions to select ADP500 derivatives with spontaneous mutations in *pcaH* or *pcaG*, strains with no change in phenotype apparent after replacement of the Δ *pcaBDK1* deletion on the chromosome with wild-type DNA were also isolated, consistent with the presence of a *vanK* mutation. Also consistent with the use of 1 mM protocatechuate expanding the potential mutational targets compared to selection with 3 mM protocatechuate, the former conditions consistently yielded significantly more spontaneous mutant derivatives per ADP500 CFU.

In another study, ADP500 derivatives were selected for resistance to 3 mM protocatechuate during growth with succinate at 22°C rather than 37°C (11). During these experiments, it was noticed that consistently more than half of the protocatechuate-resistant colonies first appearing on the selective plate, when subsequently tested, could not grow in the presence of 5 mM *p*-hydroxybenzoate at either 22 or 37°C. Because this anomalous resistance to protocatechuate but not the up-

stream metabolite *p*-hydroxybenzoate was suggestive of the phenotype resulting from *vanK* inactivation, 46 such strains had been saved. Of these 46 strains, 25 were resistant to 3 mM vanillate, implying that structural *van* genes as well as *vanK* had been inactivated by a deletion mutation (Fig. 2). The remaining 21 strains, in which *vanK* alone appeared to be mutant, were purified, and cell lysates of these strains were used in marker rescue experiments with three recipients: ADP7577, ADP7582, and ADP7587 (Table 1). Lysates of 13 strains could correct all but the Δ *vanK1103* mutation in ADP7582; because these strains probably also carried Δ *vanK1103*, they were not analyzed further.

Each of the eight remaining strains, lysates of which could correct the *vanK* mutation in all three recipients, appeared to have a new mutation in *vanK*. As before, the Δ *pcaBDK1* deletion in these mutants was replaced on the chromosome with Δ *pcaK859*, generating ADP7602 to ADP7609 (Table 1), and the *vanK* mutation was localized by marker rescue with plasmids pZR139, pZR143, and pZR144. DNA sequencing of the relevant *vanK* regions revealed three cases of point mutations producing amino acid substitutions in VanK: Gly₁₅₄Trp in VanK7604, Gln₂₁₉Stop in VanK7608, and Arg₃₇₈Pro in VanK7609 (Fig. 3). One strain had a 150-bp deletion (Δ *vanK7607*) flanked by the 4-bp sequence ATTA, but the deletion left both repeats present (Fig. 3). Three strains had Δ *vanK7602* in which 96 bp is deleted, including one flanking 8-bp repeat of the sequence GCTGGCGT (Fig. 3). Intriguingly, a sequence identical in seven of eight positions is involved in a spontaneous deletion mutation, *mucK51* (77), in an *Acinetobacter* gene encoding a permease closely related to VanK (Fig. 4), and the 3' endpoint of the deletion is in the equivalent position of both genes.

PCR amplification of DNA containing the remaining mutation, *vanK7603*, indicated an insertion of 1.2 kb, and sequencing confirmed the presence of the *Acinetobacter* insertion sequence IS1236 (27). Insertion of IS1236 in the direction of *vanK* transcription generated a 3-bp target site duplication (Fig. 3). This insertion behavior, characteristic of the IS3 family (24), was also seen in previous studies in the two regulator genes *pobR* (27) and *pcaU* (11), in contrast to three of four insertions in the *pcaH* structural gene (28). Tn5613, a newly discovered compound transposon with identical copies of IS1236 at either end, contributes to spontaneous mutation in *Acinetobacter vanA* and *vanB* and also generates a 3-bp target site duplication (69).

Overlapping specificities of VanK and PcaK revealed during catabolism of protocatechuate, *p*-hydroxybenzoate, and quinate. To systematically identify compounds in the β -ketoadipate pathway (Fig. 1) for which VanK and PcaK had overlapping specificities, ADP7592 (Δ *vanK1103* Δ *pcaBDK1*) was tested for growth with 10 mM succinate at two temperatures (37 and 22°C) in the presence of various concentrations (0.1 to 5 mM) of protocatechuate, *p*-hydroxybenzoate, quinate, or vanillate. ADP500 (Δ *pcaBDK1*) was sensitive to these compounds under all conditions and concentrations tested, indicating that *pcaK* inactivation alone was not sufficient to provide resistance. At 37°C, besides being resistant to 1 mM protocatechuate, ADP7592 grew in the presence of 5 mM quinate or 0.1 mM *p*-hydroxybenzoate. Higher concentrations of *p*-hydroxybenzoate of vanillate at even 0.1 mM were toxic, consistent with the phenotype observed during isolation of other mutants, including those later shown to have larger deletions within *vanK* (Fig. 3). Emphasizing the importance of growth temperature, ADP7592 was resistant to 3 mM protocatechuate during growth at 22°C but not 37°C. To test if inactivation of *vanK* alone was sufficient for any resistance, strain ADP7593

(Δ *vanK1103* Δ *pcaBD1*) was constructed (see Materials and Methods). Growth tests with ADP7593 showed that no protection against toxicity was provided only by *vanK* inactivation; therefore, the observed resistance phenotypes required inactivation of both *vanK* and *pcaK*.

In a genetic background in which *pcaB* was blocked, the enhanced resistance described above in strains in which both *vanK* and *pcaK* were inactivated suggested that these two genes could have overlapping roles in growth with protocatechuate, *p*-hydroxybenzoate, and quinate. More direct evidence for these roles came from growth tests with strains in which the only mutations were in *vanK* and *pcaK*. In strains with the Δ *pcaK859* mutation in combination with Δ *vanK7602* or any of the other sequenced *vanK* mutations (Fig. 3), growth on plates with 5 mM quinate or 3 mM protocatechuate as sole carbon and energy source was barely detectable after overnight incubation. Inactivation of both *vanK* and *pcaK* was required for this phenotype: transformation of the double-mutant strains with cloned DNA containing either *vanK* or *pcaK* yielded recombinants able to grow with quinate or protocatechuate at a level disguising the remaining mutation. In contrast, overnight growth with *p*-hydroxybenzoate or vanillate, either on plates or in liquid culture, was not clearly impaired for strains in which both *vanK* and *pcaK* were dysfunctional. This finding is consistent with the observation that inactivation of the two permease genes in ADP7592 did not prevent the toxic accumulation of carboxymuconate during growth in the presence of vanillate and conferred resistance to *p*-hydroxybenzoate only when supplied at a low concentration (0.1 mM). Furthermore, these same phenotypes were also observed in an engineered strain (ADP7614) in which Δ *pcaK859* was combined with an insertion into the *SphI* site in *vanK* (69) of the chloramphenicol resistance gene from pCAT19 (22).

VanK PcaK double mutants exhibited an even stronger phenotype with protocatechuate and quinate in liquid culture. Strain ADP7602 (Δ *vanK7602* Δ *pcaK859*) was tested since phenotypic revertants were readily obtained from other strains, especially those with Δ *vanK1103* (Fig. 3). Indeed, the first attempt to sequence this latter mutation in ADP7585 and ADP7589 (Table 1) gave results consistent with a mixed population of cells, including those in which Δ *vanK1103* had directly reverted. CFU from a 5-ml culture inoculated with approximately 10^7 stationary-phase cells of succinate-grown ADP7602 and provided either with 3 mM protocatechuate or 3 mM quinate consistently had decreased 10- or 100-fold, respectively, after 24 h of shaking incubation. Cultures with protocatechuate incubated for an additional 24 h would generally become turbid, consistent with another transport system in the cell having been activated by mutation. In contrast, cultures with quinate continued to decrease in CFU, and no growth was ever observed even after prolonged incubation (14 days).

Extracellular accumulation of protocatechuate during growth in the presence of quinate of strains blocked in *vanK* and *pcaK*. That the substrate specificity of VanK and PcaK might overlap to include the structurally similar aromatic compounds protocatechuate and *p*-hydroxybenzoate was unsurprising, but the strong phenotype associated with growth with the hydroaromatic compound quinate was unexpected (Fig. 1). A clue that the phenotype with quinate was indirect came from the observation that strains without a functional *vanK* and *pcaK*, growing on succinate in the presence of quinate, stained the medium a dramatic purple color characteristic of protocatechuate accumulation. This extracellular accumulation could be readily explained if conversion of quinate to protocatechuate in *Acinetobacter* takes place in the periplasm, outside the inner cell membrane (Fig. 5). Such compartmentation may

FIG. 5. Predicted periplasmic localization of quinate and shikimate catabolism to protocatechuate. Genetic evidence from this study suggests that the sequential action of the QuiA membrane-bound quinate/shikimate dehydrogenase (17), the QuiB dehydroquinone dehydratase (18), and the QuiC dehydroshikimate dehydratase (18) produces protocatechuate in the periplasm. Protocatechuate thus formed could then be transported across the inner membrane (dashed horizontal lines) into the cytoplasm by the PcaK and VanK permeases. Such compartmentation would reduce competition for intermediates that are common to both the catabolic pathway and the biosynthetic pathway for generation of aromatic amino acids. The putative porin QuiX (18) may mediate entry of quinate and shikimate into the periplasm from outside the cell.

cause a further obstacle to growth of *vanK* *pcaK* double mutants with quinate since the final step in conversion of quinate to protocatechuate, catalyzed by QuiC (Fig. 1), is susceptible to feedback inhibition by protocatechuate in at least one member of the genus *Acinetobacter* (74).

Extracellular accumulation of protocatechuate can reach concentrations high enough to significantly affect neighboring cells and was detected by monitoring growth of ADP603 (Δ qui1 Δ pcaBDK1) on a plate with 10 mM succinate and 5 mM quinate (Fig. 6). Deletion in ADP603 of *qui* genes (17), required for conversion of quinate to protocatechuate, prevents

FIG. 6. Detection of extracellular protocatechuate generated by mutant strains growing in the presence of quinate. A 20- μ l aliquot of an LB culture of each strain was spotted onto a plate with 10 mM succinate and 5 mM quinate and incubated overnight at 37°C. In the top and bottom rows are cells of ADP603 (Δ qui1 Δ pcaBDK1) added simultaneously (top row) or 5 h after (bottom row) strains in the middle row (from left to right): ADP1 (wild type), ADP859 (Δ pcaK859), ADP7618 (Δ vanK1103), and ADP7577 (Δ pcaK859 Δ vanK1103). Deletion of quinate genes in ADP603 prevents the toxic accumulation of carboxymuconate during growth of this strain in the presence of quinate but not protocatechuate (Fig. 1). Growth of ADP603 can therefore serve as a detector of protocatechuate diffusing from adjacent cells of mutant strains impaired to various degrees in transport of protocatechuate across the inner cell membrane after its generation from quinate in the periplasm (Fig. 5). The speckling observed in the spot of ADP7577 cells is due to reversion of Δ vanK1103.

the toxic accumulation of carboxymuconate during growth of this strain in the presence of quinate (Fig. 1). Protocatechuate itself, however, remains toxic to ADP603. Growth of ADP603, when adjacent to cells of ADP7577 (Δ vanK1103 Δ pcaK859), was drastically inhibited as expected if protocatechuate was accumulating extracellularly during growth of ADP7577 in the presence of quinate (Fig. 6). This effect was also produced, although less intensely, by protocatechuate diffusing from cells in which either *vanK* or *pcaK* was dysfunctional (Fig. 6).

Positive selection of strains with a mutation in *pcaK*. In strains without PcaB function (Fig. 1), resistance to the toxic accumulation of carboxymuconate produced during growth in the presence of protocatechuate requires inactivation of both *vanK* and *pcaK*. This requirement was exploited to select strains derived from ADP500 (Δ pcaBDK1), each with a spontaneous mutation in *vanK* (Table 1; Fig. 3). The success of this selection in turn suggested that in the appropriate genetic background, *pcaK* mutants could be isolated by positive selection as well. Previously, PCR-generated mutations had been introduced into the *Acinetobacter* chromosome by natural transformation facilitating genetic analysis of the structural genes *pcaH* and *pcaG* (12, 43) and the regulatory genes, *pobR* and *pcaU* (43, 44). Applying this technique to *pcaK*, transformation of ADP7593 (Δ vanK1103 Δ pcaBD1) with PCR-amplified DNA containing the *pcaK* gene was followed by selection for growth with succinate at 22°C in the presence of 1 mM protocatechuate. Consistent with the introduction of PCR-generated mutations into *pcaK* on the chromosome, transformants of ADP7593 that were resistant to protocatechuate appeared at a frequency over 200-fold above the spontaneous mutation frequency of approximately 1 in 10⁶ CFU.

Intragenic suppression of a *vanK* point mutation. The Gly₇₁Asp substitution in VanK1113 (Fig. 3) changes an amino acid residue conserved in all eight transport proteins in Fig. 4.

This conservation suggests that Gly₇₁ is particularly important for VanK function. Therefore, to gain insight into how structure influences function in VanK, PCR mutagenesis was again used, this time to obtain intragenic suppressors of *vanK1113*. PCR amplification across *vanK* but excluding the *vanK1113* locus near the start of the gene produced DNA that yielded recombinants of ADP7587 (*vanK1113* Δ *pcaK859*) that could grow at 30°C like wild-type cells with quinate. DNA sequencing of two such recombinants revealed in one of the strains, in addition to *vanK1113*, two new mutations, *vanK7610* and *vanK7611*, causing Phe₂₆₉Ser and Phe₂₇₁Ser substitutions, respectively (Fig. 3). In the other recombinant, one new mutation, *vanK7612*, produced a Gly₂₉₄Arg substitution (Fig. 3).

DISCUSSION

Multiple spontaneous mutations in strains derived from a single genetic selection. The first evidence for the existence of *vanK* came from the detection of two spontaneous mutations in ADP500-derived strains selected for resistance to the toxic accumulation of carboxymuconate produced during growth in the presence of protocatechuate. One of these mutations was in *pcaH* or *pcaG*, structural genes for protocatechuate 3,4-dioxygenase, and the other inactivated *vanK*, encoding a putative transport system able to act on protocatechuate. A remarkably similar result was described in two recent *Acinetobacter* studies involving a parallel branch of the β -ketoadipate pathway. Selection for resistance to the toxic internal accumulation of muconate also generated strains with two spontaneous mutations, one in a structural gene for benzoate or anthranilate degradation and one inactivating the muconate transporter MucK (5, 77). The transporter mutation in this case, although not sufficient to allow growth (77), could have been beneficial by blocking the uptake of muconate accumulating extracellularly at the onset of selection from the lawn of cells of the parental strain (5, 23, 77). In the case of *vanK*, multiple mutations may have been an artifact of the selection procedure: in at least one ADP500 derivative already resistant to protocatechuate, inactivation of *vanK* appeared to confer additional resistance and was identified by its forming a relatively large colony during purification on a plate with selective media. The exact nature of the additional resistance provided by the block in protocatechuate uptake is unclear but may reflect the relative toxicity of this aromatic compound (62).

The unexpected spontaneous transport protein mutations in *Acinetobacter* described above illustrate the potential complexity of genetic selections. This complexity is consistent with the suggestion that sequential mutations can confer incremental benefits during nonlethal genetic selection, although only one mutation may ultimately be detected (25, 47). Furthermore, the results of this study highlight the influence of selection conditions and the genetic background of the starting strain on the range of genetic loci revealed by mutation, a conclusion that is probably particularly relevant to analyses of transport proteins (3).

A hot spot for frameshift mutation in a poly(G) tract in *vanK*. In the 10 *Acinetobacter* strains analyzed by DNA sequencing that had a spontaneous mutation in both *vanK* and *pcaH* or *pcaG*, 8 contained Δ *vanK1103*, a single-nucleotide deletion in a string of 6 G residues (Fig. 3). When strains in which only *vanK* had acquired a mutation were selected, a greater variety of mutations emerged, but still the majority (13 of 21) contained Δ *vanK1103*. The frequent selection of Δ *vanK1103* identifies the mononucleotide repeat as a hot spot for frameshift mutation. Furthermore, this poly(G) tract is not conserved in the genes for the five permeases most closely

related to VanK, raising the possibility that the genetic instability reflected by Δ *vanK1103* may be of adaptive significance to *Acinetobacter* in the soil environment. High-frequency frameshifting in a poly(A) tract in a gene encoding an ABC transporter component (72) has been suggested to generate alternative transport capabilities in mycoplasmas organisms with apparently limited transport systems (72), and phase variation due to reversible frameshifting in a poly(G) tract has been described for several bacterial genes involved in pathogenesis (6, 10, 38). The mutability of these contingency genes (55) can facilitate adaptation to rapidly changing and unpredictable environmental challenges (15, 55, 67), and this may apply to *Acinetobacter vanK* as well.

Insight into VanK structure and function from second-site suppressor mutations. By demanding growth of ADP500 (Δ *pcaABDK1*) on succinate at 22°C in the presence of 3 mM protocatechuate, strains with secondary spontaneous mutations in *vanK* were isolated. Subsequent tests of lysates of these spontaneous mutants in marker rescue experiments rapidly identified strains with mutations not caused by the Δ *vanK1103* frameshift hot spot. By using this simple procedure, a VanK missense mutation was found in three strains (Fig. 3): two mutations altered a glycine, Gly₇₁Asp (VanK1113) and Gly₁₅₄Trp (VanK7604), the former introducing a charged residue into a putative membrane-spanning helix, and the third mutation was a substitution with a proline residue, Arg₃₇₈Pro (VanK7609). A combination of selection with transformation-facilitated PCR mutagenesis (12, 43, 44), successfully tested on chromosomal *pcaK* in this study, will allow the isolation of a large collection of strains with point mutations useful for identifying amino acid residues critical for structure and function of the two homologous *Acinetobacter* transport proteins, VanK and PcaK.

A signature sequence for the aromatic acid transporter family within the major facilitator superfamily includes most of the residues forming predicted transmembrane segment 2 (61). A completely conserved glycine residue in this sequence is changed by the Gly₇₁Asp mutation in VanK1113 (Fig. 3). PCR mutagenesis of *vanK* DNA encoding putative transmembrane helices 4 to 12 in this mutant led to the isolation of two phenotypic revertants, one with a double mutation, Phe₂₆₉Ser Phe₂₇₁Ser, in helix 7 and one with a Gly₂₉₄Arg substitution adjacent to helix 8 (Fig. 7). Substitution of a glycine residue in transmembrane segment 2 that inactivates LacY, the prototypic member of the major facilitator superfamily, is suppressed by second-site mutations, including ones in the second half of the protein in transmembrane segments 7 and 8 (37). In combination with biochemical data, this finding led to a model of LacY function whereby substrate binding generates a global conformation change propagated from helix 8 and mediated by helix 2 at the interface of the two halves of the protein (37, 40). The congruence of the LacY suppression analysis with the preliminary VanK results is consistent with the two proteins having a similar mechanism of action. This shared mechanism may be underlaid by the fact that all the members of the major facilitator superfamily are believed to be derived from a six-transmembrane-helix protein, the gene for which underwent an ancient tandem duplication (51, 61).

Evidence that quinate is converted to protocatechuate in the periplasm. In *Acinetobacter* strain ADP1, the hydroaromatic compound quinate (Fig. 1) is converted to protocatechuate by the sequential action of three enzymes (QuiA, QuiB, and QuiC) encoded by genes in the *pca-qui-pob* supraoperonic cluster (17, 18, 30, 35). The reactions catalyzed by the catabolic enzymes QuiB and QuiC can interfere with the same reactions in the opposite physiological direction, performed by enzymes

FIG. 7. Intragenic suppression of VanK1113 (Gly₇₁Asp). Transformation-facilitated PCR mutagenesis in ADP7587 (*vanK1113* Δ *pcaK859*) generated two strains with second-site suppressors of VanK1113, a Gly₇₁Asp substitution in VanK transmembrane helix 2. ADP7610 had two suppressors in transmembrane helix 7, Phe₂₆₉Ser and Phe₂₇₁Ser, and ADP7612 had one suppressor adjacent to transmembrane helix 8, Gly₂₉₄Arg (Fig. 3).

in the shikimate pathway for aromatic amino acid biosynthesis (18) (Fig. 5). In fungi, channeling of intermediates in an aggregate of the shikimate pathway biosynthetic enzymes prevents access by catabolic enzymes during growth with quinate (29, 46). Evidence for physical segregation of the two classes of enzymes has also been described in *Acinetobacter* (18, 74). Further evidence presented here supports catabolism of quinate to protocatechuate taking place in the periplasm: *pcaK vanK* double mutants severely impaired in growth with protocatechuate because of their inability to transport this compound across the inner membrane into the cytoplasm also are severely impaired in growth with quinate, although quinate appears to be efficiently converted to extracellular protocatechuate (Fig. 6). Periplasmic localization of QuiABC is consistent with the amino acid sequence of QuiA (17), which identifies it as a member of a family of membrane-associated, PQQ-dependent dehydrogenases (16), as well as a putative leader peptide in the N terminus of QuiB (18) which could mediate its export out of the cytoplasm.

Spontaneous mutant derivatives of ADP500 resistant to the toxic accumulation of carboxymuconate during growth in the presence of quinate never contained mutations confined to *qui* genes and were always blocked at the level of protocatechuate degradation (19). This observation can now be explained by the ease with which protocatechuate accumulates extracellularly after its generation in the periplasm from quinate. This is particularly true in strains blocked in PcaK such as ADP500 (Fig. 6) but is probably also relevant to the wild-type strain growing in the more natural condition of multiple carbon sources: significant amounts of protocatechuate accumulated when *Acinetobacter* was provided with shikimate, another substrate of QuiA (Fig. 5), simultaneously with three other carbon sources (23). This accumulation may reach local extracellular concentrations that are toxic to some competing soil microorganisms (Fig. 6). On the other hand, this "overflow metabolism" may mediate mutually beneficial cross-feeding (42). Similarly, bacterial growth on aromatic compounds can lead to extracellular accumulation of β -ketoadipate (23, 41), a chemotactant that has been suggested to mediate the formation of "catabolic consortia" (63) of soil bacteria. In this light, reversible inactivation of *Acinetobacter vanK* by mutation at the frameshift hot spot may underlie a switch in membrane permeability important for cell-cell interactions.

Membership of *Acinetobacter* QuiA in a family of PQQ-dependent membrane-bound dehydrogenases suggests a further link between oxidation of quinate, a compound ubiquitous in the soil environment (36), and active transport. The single step of oxidation of glucose to gluconate by a dehydrogenase closely related to QuiA has been shown in several organisms to generate a proton motive force which could efficiently power secondary transport systems (75). Conversion of quinate to dehydroquininate may likewise power transport by PcaK and

VanK, facilitating scavenging of aromatic compounds from the environment even in the absence of growth. Such a role may explain why transcription of *Acinetobacter quiA* appears, at least in part, to be independently regulated (18). Also, as has been suggested for gluconate production from glucose in *Pseudomonas* (68), periplasmic oxidation of quinate by *Acinetobacter* cells may serve to rapidly remove this compound from access by competing soil microorganisms.

Overlapping specificity of the VanK and PcaK transport proteins. The *pca* operon encodes the PcaK transport protein together with all the enzymes for conversion of protocatechuate to citric acid cycle intermediates (45). The role of PcaK in protocatechuate transport can be masked by the overlapping specificity of VanK, encoded by a gene distant on the chromosome from the *pca-qui-pob* supraoperonic cluster and part of a separate cluster of genes for conversion of vanillate to protocatechuate (69) (Fig. 2). Despite the location of *vanK*, no evidence indicating that the encoded protein transports vanillate has yet been found, although the activities of yet to be identified transporters may well mask such a role. The full spectrum of compounds that can be transported by PcaK and VanK remains to be elucidated.

The results of this study, consistent with studies of *P. putida* PcaK (34, 56), indicate that both VanK and PcaK can mediate uptake of protocatechuate and *p*-hydroxybenzoate: inactivation of both proteins severely impairs growth with protocatechuate and, in cells lacking PcaB function, prevents toxic accumulation of carboxymuconate during growth in the presence of low levels of *p*-hydroxybenzoate. This latter phenotype should allow the genetic dissection of transport of these two compounds by *Acinetobacter* PcaK. After transformation-facilitated PCR mutagenesis of chromosomal *pcaK* in ADP7593 (Δ *vanK1103* Δ *pcaBD1*), recombinant cells selected for resistance to protocatechuate can be screened for lack of resistance to an appropriate concentration of *p*-hydroxybenzoate. This procedure should identify strains with a PcaK amino acid substitution in which only protocatechuate uptake is significantly impaired.

The requirement for either VanK or PcaK for efficient growth with protocatechuate may support the idea that transport systems play a fundamental role in regulating cytoplasmic levels of metabolites that are also inducers of gene expression (58, 59). This concept has been recently emphasized (2) following studies of aromatic catabolism which revealed linkage on the chromosome of a gene for a PcaK family member and a gene for a regulatory protein predicted to respond to the aromatic substrate of the transporter. In this light, the apparent redundancy of VanK and PcaK is consistent with the need to concentrate the same inducer-metabolite in two spatially segregated cellular compartments. The most likely candidate for this inducer is protocatechuate, given that it activates *pca* operon expression (26, 44) and, although it awaits determina-

tion in *Acinetobacter*, it also appears to induce expression of *vanA* and *vanB* in *Pseudomonas* (76).

Facilitating the channeling of protocatechuate after it enters the cytoplasm, the gene clusters containing *vanK* and *pcaK* may become associated with the cytoplasmic membrane by coupling of transcription of these genes with translation and membrane insertion of the encoded proteins (49, 50). The substrate specificity of the transport protein encoded in each gene cluster would thereby become pivotal: capable of either preventing cross talk between biochemical pathways or conversely, of efficiently mediating such interactions (66). A more general role in the β -ketoadipate pathway of metabolic channeling by enzyme complexes (21, 53, 60, 71) has been suggested to underly the supraoperonic clustering (23) of genes in *Acinetobacter* as well as an explanation of the different effects caused by an inducer added extracellularly versus made endogenously as described for both *Acinetobacter* (23) and *Pseudomonas* (54).

A previous study of *Acinetobacter* examined the genetic flexibility of two closely related transcriptional activators associated with protocatechuate catabolism and found that no more than two amino acid substitutions were required for each protein to functionally replace its homolog (44). Similarly, only one amino acid substitution was required for protocatechuate dioxygenase to functionally replace catechol dioxygenase (12). This study outlines a genetic system for studying the contribution to protocatechuate catabolism of two homologous transport proteins, VanK and PcaK, already overlapping in specificity. Recognition of this overlap is particularly important given that the activity of the LacY permease can generate nongenetic heterogeneity of *lac* operon expression in *E. coli* cells exposed to suboptimal levels of inducer (57, 73). While this phenomenon provides yet another source of variability for bacteria to deal with environmental change (73), it is likely to complicate laboratory measurements of transport activity, gene expression, and growth rates in *Acinetobacter* if the activities of both VanK and PcaK are not taken into account. Genetic analysis of these two permeases in *Acinetobacter* also may complement characterization of *P. putida* PcaK, which has an additional role in chemotaxis (34, 56). Such a comparative approach may reveal new properties of aromatic acid transport systems, such as a spatial organization in the membrane (4, 70), and should be particularly useful in elucidating the molecular biology of self-identity (35).

ACKNOWLEDGMENTS

This research was supported by grants DAAG55-98-1-0232 from the Army Research Office and MCB-9603980 from the National Science Foundation. A.S. was supported by a postdoctoral fellowship from the Spanish Ministerio de Educación y Ciencia.

The overlapping specificity of VanK and PcaK was recognized thanks to the faithful recording of anomalous phenotypes of ADP500 derivatives by D. Elsemore, D. Eulberg, T. Plaggemeier, and P. West. Discussion with E. P. Greenberg prompted consideration of possible additional roles played by the membrane-associated QuiA dehydrogenase. The plate photograph was taken by W. Sacco.

REFERENCES

- Altschul, S. F., T. L. Madden, A. A. Schäfer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Barnes, M. R., W. A. Duetz, and P. A. Williams. 1997. A 3-(3-hydroxyphenyl)propionic acid catabolic pathway in *Rhodococcus globulus* PWD1: cloning and characterization of the *hpp* operon. *J. Bacteriol.* **179**:6145–6153.
- Benson, S. A., A. M. DeCloux, and J. Munro. 1991. Mutant bias in nonlethal selections results from selective recovery of mutants. *Genetics* **129**:647–658.
- Bray, D., M. D. Levin, and C. J. Morton-Firth. 1998. Receptor clustering as a cellular mechanism to control sensitivity. *Nature* **393**:85–88.
- Bundy, B. M., A. L. Campbell, and E. L. Neidle. 1998. Similarities between the *antABC*-encoded anthranilate dioxygenase and the *benABC*-encoded benzoate dioxygenase of *Acinetobacter* sp. strain ADP1. *J. Bacteriol.* **180**:4466–4474.
- Carroll, P. A., K. T. Tashima, M. B. Rogers, V. J. DiRita, and S. B. Calderwood. 1997. Phase variation in *tcpH* modulates expression of the ToxR regulon in *Vibrio cholerae*. *Mol. Microbiol.* **25**:1099–1111.
- Clayton, R. A., O. White, K. A. Ketchum, and J. C. Venter. 1997. The first genome from the third domain of life. *Nature* **387**:459–462.
- Coco, W. M., and L. N. Ornston. 1997. *Acinetobacter* PcaK is a multispecificity active facilitator of aromatic acid transport, abstr. K-81, p. 355. In Abstracts of the 97th General Meeting of the American Society for Microbiology 1997. American Society for Microbiology, Washington, D.C.
- Collier, L. S., N. N. Nichols, and E. L. Neidle. 1997. *benK* encodes a hydrophobic permease-like protein involved in benzoate degradation by *Acinetobacter* sp. strain ADP1. *J. Bacteriol.* **179**:5943–5946.
- Danaher, R. J., J. C. Levin, D. Arking, C. L. Burch, R. Sandlin, and D. C. Stein. 1995. Genetic basis of *Neisseria gonorrhoeae* lipooligosaccharide antigenic variation. *J. Bacteriol.* **177**:7275–7279.
- D'Argenio, D. A., A. Segura, P. V. Bünz, and L. N. Ornston. Unpublished data.
- D'Argenio, D. A., M. W. Vetting, D. H. Ohlendorf, and L. N. Ornston. Unpublished data.
- DiMarco, A. A., B. A. Averhoff, E. E. Kim, and L. N. Ornston. 1993. Evolutionary divergence of *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase in an *Acinetobacter calcoaceticus* strain well-suited for genetic analysis. *Gene* **125**:25–33.
- DiMarco, A. A., B. Averhoff, and L. N. Ornston. 1993. Identification of the transcriptional activator *pobR* and characterization of its role in the expression of *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase in *Acinetobacter calcoaceticus*. *J. Bacteriol.* **175**:4499–4506.
- DiRita, V. J., and J. J. Mekalanos. 1989. Genetic regulation of bacterial virulence. *Annu. Rev. Genet.* **23**:455–482.
- Duine, J. A. 1991. Quinoproteins: enzymes containing the quinoid cofactor pyrroloquinoline quinone, topaquinone or tryptophan-tryptophan quinone. *Eur. J. Biochem.* **200**:271–284.
- Elsemore, D. A., and L. N. Ornston. 1994. The *pca-pob* supraoperonic cluster of *Acinetobacter calcoaceticus* contains *quiA*, the structural gene for quinate-shikimate dehydrogenase. *J. Bacteriol.* **176**:7659–7666.
- Elsemore, D. A., and L. N. Ornston. 1995. Unusual ancestry of dehydratases associated with quinate catabolism in *Acinetobacter calcoaceticus*. *J. Bacteriol.* **177**:5971–5978.
- Elsemore, D. A., P. West, and L. N. Ornston. Personal communication.
- Ferrández, A., J. L. García, and E. Díaz. 1997. Genetic characterization and expression in heterologous hosts of the 3-(3-hydroxyphenyl)propionate catabolic pathway of *Escherichia coli* K-12. *J. Bacteriol.* **179**:2573–2581.
- Fraenkel, D. G. 1992. Genetics and intermediary metabolism. *Annu. Rev. Genet.* **26**:159–177.
- Fuqua, W. C. 1992. An improved chloramphenicol resistance gene cassette for site-directed marker replacement mutagenesis. *BioTechniques* **12**:223–225.
- Gaines, G. L., III, L. Smith, and E. L. Neidle. 1996. Novel nuclear magnetic resonance spectroscopy methods demonstrate preferential carbon source utilization by *Acinetobacter calcoaceticus*. *J. Bacteriol.* **178**:6833–6841.
- Galas, J. G., and M. Chandler. 1989. Bacterial insertion sequences, p. 109–162. In Edited by D. E. Berg and M. M. Howe (ed.), *Mobile DNA*. American Society for Microbiology, Washington, D.C.
- Galitski, T., and J. R. Roth. 1996. A search for a general phenomenon of adaptive mutability. *Genetics* **143**:645–659.
- Gerischer, U., A. Segura, and L. N. Ornston. 1997. PcaU, transcriptional activator of genes for protocatechuate utilization in *Acinetobacter*. *J. Bacteriol.* **180**:1512–1524.
- Gerischer, U., D. A. D'Argenio, and L. N. Ornston. 1996. IS1236, a newly discovered member of the IS3 family, exhibits varied patterns of insertion into the *Acinetobacter calcoaceticus* chromosome. *Microbiology* **142**:1825–1831.
- Gerischer, U., and L. N. Ornston. 1995. Spontaneous mutations in *pcaH* and *-G*, structural genes for protocatechuate 3,4-dioxygenase in *Acinetobacter calcoaceticus*. *J. Bacteriol.* **177**:1336–1347.
- Giles, N. H., C. W. H. Partridge, S. I. Ahmed, and M. E. Case. 1967. The occurrence of two dehydroquinases in *Neurospora crassa*, one constitutive and one inducible. *Proc. Natl. Acad. Sci. USA* **58**:1930–1937.
- Gralton, E. M., A. L. Campbell, and E. L. Neidle. 1997. Directed introduction of DNA cleavage sites to produce a high-resolution genetic and physical map of the *Acinetobacter* sp. strain ADP1 (BD413) chromosome. *Microbiology* **143**:1345–1357.
- Hartnett, C., E. L. Neidle, K.-L. Ngai, and L. N. Ornston. 1990. DNA sequence of genes encoding *Acinetobacter calcoaceticus* protocatechuate 3,4-dioxygenase: evidence indicating shuffling of genes and of DNA sequences within genes during their evolutionary divergence. *J. Bacteriol.* **172**:956–966.
- Hartnett, G. B. 1993. Ph.D. thesis. Yale University, New Haven, Conn.
- Hartnett, G. B., B. Averhoff, and L. N. Ornston. 1990. Selection of *Acinetobacter calcoaceticus* mutants deficient in the *p*-hydroxybenzoate hydroxylase

- gene (*pobA*), a member of a supraoperonic cluster. *J. Bacteriol.* **172**:6160–6161.
34. Harwood, C. S., N. N. Nichols, M.-K. Kim, J. L. Ditty, and R. E. Parales. 1994. Identification of the *pcaRKF* gene cluster from *Pseudomonas putida*: involvement in chemotaxis, biodegradation, and transport of 4-hydroxybenzoate. *J. Bacteriol.* **176**:6479–6488.
 35. Harwood, C. S., and R. E. Parales. 1996. The β -ketoadipate pathway and the biology of self-identity. *Annu. Rev. Microbiol.* **50**:553–590.
 36. Hawkins, A. R., H. K. Lamb, J. D. Moore, I. G. Charles, and C. F. Roberts. 1993. The pre-chorismate (shikimate) and quinate pathways in filamentous fungi: theoretical and practical aspects. *J. Gen. Microbiol.* **139**:2891–2899.
 37. Jessen-Marshall, A. E., N. J. Parker, and R. J. Brooker. 1997. Suppressor analysis of mutations in the loop 2-3 motif of lactose permease: evidence that glycine-64 is an important residue for conformational changes. *J. Bacteriol.* **179**:2616–2622.
 38. Jonsson, A.-B., G. Nyberg, and S. Normark. 1991. Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. *EMBO J.* **10**:477–488.
 39. Juni, E., and A. Janick. 1969. Transformation of *Acinetobacter calcoaceticus* (*Bacterium anitratum*). *J. Bacteriol.* **98**:281–288.
 40. Kaback, H. R. 1997. A molecular mechanism for energy coupling in a membrane transport protein, the lactose permease of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **94**:5539–5543.
 41. Kilby, B. A. 1951. The formation of β -ketoadipic acid by bacterial fission of aromatic rings. *Biochem. J.* **49**:671–674.
 42. Koch, A. L. 1997. Microbial physiology and ecology of slow growth. *Microbiol. Mol. Biol. Rev.* **61**:305–318.
 43. Kok, R. G., D. A. D'Argenio, and L. N. Ornston. 1997. Combining localized PCR mutagenesis and natural transformation in direct genetic analysis of a transcriptional regulator gene, *pobR*. *J. Bacteriol.* **179**:4270–4276.
 44. Kok, R. G., D. A. D'Argenio, and L. N. Ornston. 1998. Mutation analysis of *PobR* and *PcaU*, closely related transcriptional activators in *Acinetobacter*. *J. Bacteriol.* **180**:5058–5069.
 45. Kowalchuk, G. A., G. B. Hartnett, A. Benson, J. E. Houghton, K.-L. Ngai, and L. N. Ornston. 1994. Contrasting patterns of evolutionary divergence within the *Acinetobacter calcoaceticus* *pca* operon. *Gene* **146**:23–30.
 46. Lamb, H. K., J. P. T. W. van den Hombergh, G. H. Newton, J. D. Moore, C. F. Roberts, and A. R. Hawkins. 1992. Differential flux through the quinate and shikimate pathways. Implications for the channeling hypothesis. *Biochem. J.* **284**:181–187.
 47. Lenski, R. E., M. Slatkin, and F. J. Ayala. 1989. Mutation and selection in bacterial populations: alternatives to the hypothesis of directed mutation. *Proc. Natl. Acad. Sci. USA* **86**:2775–2778.
 48. Leveau, J. H., A. J. B. Zehnder, and J. R. van der Meer. 1998. The *tfdK* gene product facilitates uptake of 2,4-dichlorophenoxyacetate by *Ralstonia eutropha* JMP134(pJP4). *J. Bacteriol.* **180**:2237–2243.
 49. Lodge, J. K., T. Kazic, and D. E. Berg. 1989. Formation of supercoiling domains in plasmid pBR322. *J. Bacteriol.* **171**:2181–2187.
 50. Lynch, A. S., and J. C. Wang. 1993. Anchoring of DNA to the bacterial cytoplasmic membrane through cotranscriptional synthesis of polypeptides encoding membrane proteins or proteins for export: a mechanism of plasmid hypernegative supercoiling in mutants deficient in DNA topoisomerase I. *J. Bacteriol.* **175**:1645–1655.
 51. Maiden, M. C. J., E. O. Davis, S. A. Baldwin, D. C. M. Moore, and P. J. F. Henderson. 1987. Mammalian and bacterial sugar transport proteins are homologous. *Nature* **325**:641–643.
 52. Marger, M. D., and M. H. Saier, Jr. 1993. A major superfamily of transmembrane facilitators that catalyze uniport, symport, and antiport. *Trends Biochem. Sci.* **18**:13–20.
 53. Mathews, C. K. 1993. The cell-bag of enzymes or network of channels? *J. Bacteriol.* **175**:6377–6381.
 54. Meagher, R. B., G. M. McCorkle, M. K. Ornston, and L. N. Ornston. 1972. Inducible uptake system for β -carboxy-*cis*-muconate in a permeability mutant of *Pseudomonas putida*. *J. Bacteriol.* **111**:465–473.
 55. Moxon, E. R., P. B. Rainey, M. A. Nowak, and R. E. Lenski. 1994. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr. Biol.* **4**:24–33.
 56. Nichols, N. N., and C. S. Harwood. 1997. *PcaK*, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. *J. Bacteriol.* **179**:5056–5061.
 57. Novick, A., and M. Weiner. 1957. Enzyme induction as an all-or-none phenomenon. *Proc. Natl. Acad. Sci. USA* **43**:553–566.
 58. Ondrako, J. M., and L. N. Ornston. 1980. Biological distribution and physiological role of the β -ketoadipate transport system. *J. Gen. Microbiol.* **120**:199–209.
 59. Ornston, L. N., and D. Parke. 1977. The evolution of induction mechanisms in bacteria: insights derived from the study of the β -ketoadipate pathway. *Curr. Top. Cell Regul.* **12**:209–262.
 60. Ovádi, J. 1991. Physiological significance of metabolic channeling. *J. Theor. Biol.* **152**:1–22.
 61. Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr. 1998. Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* **62**:1–34.
 62. Parke, D., and L. N. Ornston. 1984. Nutritional diversity of *Rhizobiaceae* revealed by auxanography. *J. Gen. Microbiol.* **130**:1743–1750.
 63. Parke, D., M. Rivelli, and L. N. Ornston. 1985. Chemotaxis to aromatic and hydroaromatic acids: comparison of *Bradyrhizobium japonicum* and *Rhizobium trifoli*. *J. Bacteriol.* **163**:417–422.
 64. Paulsen, I. T., M. K. Sliwinski, and M. H. Saier, Jr. 1998. Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. *J. Mol. Biol.* **277**:573–592.
 65. Plaggemeier, T., and D. Eulberg. Unpublished data.
 66. Reid, C. J., and P. S. Poole. 1998. Roles of DctA and DctB in signal detection by the dicarboxylic acid transport system of *Rhizobium leguminosarum*. *J. Bacteriol.* **180**:2660–2669.
 67. Robertson, B. D., and T. F. Meyer. 1992. Genetic variation in pathogenic bacteria. *Trends Genet.* **8**:422–427.
 68. Schleissner, C., A. Reglero, and J. M. Luengo. 1997. Catabolism of D-glucose by *Pseudomonas putida* U occurs via extracellular transformation into D-gluconic acid and induction of a specific gluconate transport system. *Microbiology* **143**:1595–1603.
 69. Segura, A., P. V. Büinz, D. A. D'Argenio, and L. N. Ornston. 1999. Genetic analysis of a chromosomal region containing *vanA* and *vanB*, genes required for conversion of either ferulate or vanillate to protocatechuate in *Acinetobacter*. *J. Bacteriol.* **181**:3494–3504.
 70. Shapiro, L. 1993. Protein localization and asymmetry in the bacterial cell. *Cell* **73**:841–855.
 71. Srere, P. A. 1987. Complexes of sequential metabolic enzymes. *Annu. Rev. Biochem.* **56**:89–124.
 72. Theiss, P., and K. S. Wise. 1997. Localized frameshift mutation generates selective, high-frequency phase variation of a surface lipoprotein encoded by a *Mycoplasma* ABC transporter operon. *J. Bacteriol.* **179**:4013–4022.
 73. Tolker-Nielsen, T., K. Holmström, L. Boe, and S. Molin. 1998. Non-genetic population heterogeneity studied by *in situ* polymerase chain reaction. *Mol. Microbiol.* **27**:1099–1105.
 74. Tresguerres, M. E. F., W. M. Ingledew, and J. L. Cánovas. 1972. Potential competition for 5-dehydroshikimate between the aromatic biosynthetic route and the catabolic hydroaromatic pathway. *Arch. Mikrobiol.* **82**:111–119.
 75. van Schie, B. J., K. J. Hellingwerf, J. P. van Dijken, M. G. L. Elferink, J. M. van Dijk, J. G. Kuenen, and W. N. Konings. 1985. Energy transduction by electron transfer via a pyrrolo-quinoline quinone-dependent glucose dehydrogenase in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter calcoaceticus* (var. *lwoffii*). *J. Bacteriol.* **163**:493–499.
 76. Venturi, V., F. Zennaro, G. Degrossi, B. C. Okeke, and C. V. Bruschi. 1998. Genetics of ferulic acid bioconversion to protocatechuic acid in plant-growth-promoting *Pseudomonas putida* WCS358. *Microbiology* **144**:965–973.
 77. Williams, P. A., and L. E. Shaw. 1997. *mucK*, a gene in *Acinetobacter calcoaceticus* ADP1 (BD413), encodes the ability to grow on exogenous *cis*-muconate as the sole carbon source. *J. Bacteriol.* **179**:5935–5942.