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Genetic Analysis of a Chromosomal Region Containing *vanA* and *vanB*, Genes Required for Conversion of Either Ferulate or Vanillate to Protocatechuate in *Acinetobacter*†

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VanA and VanB form an oxygenative demethylase that converts vanillate to protocatechuate in microorganisms. Ferulate, an abundant phytochemical, had been shown to be metabolized through a vanillate intermediate in several *Pseudomonas* isolates, and biochemical evidence had indicated that vanillate also is an intermediate in ferulate catabolism by *Acinetobacter*. Genetic evidence supporting this conclusion was obtained by characterization of mutant *Acinetobacter* strains blocked in catabolism of both ferulate and vanillate. Cloned *Acinetobacter vanA* and *vanB* were shown to be members of a chromosomal segment remote from a supraoperonic cluster containing other genes required for completion of the catabolism of ferulate and its structural analogs, caffeate and coumarate, through protocatechuate. The nucleotide sequence of DNA containing *vanA* and *vanB* demonstrated the presence of genes that, on the basis of nucleotide sequence similarity, appeared to be associated with transport of aromatic compounds, metabolism of such compounds, or iron scavenging. Spontaneous deletion of 100 kb of DNA containing this segment does not impede the growth of cells with simple carbon sources other than vanillate or ferulate. Additional spontaneous mutations blocking *vanA* and *vanB* expression were shown to be mediated by IS*1236*, including insertion of the newly discovered composite transposon Tn*5613*. On the whole, *vanA* and *vanB* appear to be located within a nonessential genetic region that exhibits considerable genetic malleability in *Acinetobacter*. The overall organization of genes neighboring *Acinetobacter vanA* and *vanB*, including a putative transcriptional regulatory gene that is convergently transcribed and overlaps *vanB*, is conserved in *Pseudomonas aeruginosa* but has undergone radical rearrangement in other *Pseudomonas* species.

Vanillate demethylase is a member of a superfamily of reductive dioxygenases with a broad range of activities (47). The enzymes are of interest because they provide model systems for analysis of electron transfer and because they demonstrate the consequences of modular rearrangement of catalytic domains during enzyme evolution (22).

Recent attention has focused upon vanillate as an intermediate in ferulate catabolism by *Pseudomonas* species (Fig. 1). Nucleotide sequencing of *Pseudomonas* strain ATCC 19151 genes encoding vanillate demethylase revealed open reading frames designated *vanA* and *vanB*; these genes exhibited the high G+C content characteristic of the genus (5). Examination of DNA cloned from *Pseudomonas* sp. strain HR199 revealed *vanA* and *vanB* genes on an *Eco*RI restriction fragment that also contained an open reading frame encoding vanillin dehydrogenase; the latter open reading frame was in the same operon as an open reading frame encoding a protein with amino acid sequence similarity to enoyl coenzyme A (enoyl-CoA) hydratase (57).

Mutations blocking ferulate catabolism in *Pseudomonas putida* WCS358 allowed cloning from the organism of two restriction fragments containing DNA necessary for the metabolic pathway (62). The presence of *vanA* and *vanB* in one of the clones confirmed the role of these genes in ferulate catabolism. The other clone contained open reading frames for vanillin dehydrogenase and enoyl-CoA hydratase. Analysis of enzymes associated with ferulate metabolism in *Pseudomonas fluorescens* bv. VAN103 (48) demonstrated an enoyl-CoA hydratase that also acted as a lyase cleaving the hydrated derivative of feruloyl-CoA into vanillin and acetyl-CoA (23). Thus, in at least some *Pseudomonas* species, ferulate metabolism appears to proceed by thioester formation, hydration, and cleavage, giving rise to vanillin, which is oxidized to vanillate.

Acinetobacter and fluorescent *Pseudomonas* species are the predominant representatives of the "*Pseudomonas*" group within the γ subdivision of the proteobacteria as classified by the National Center for Biotechnology Information. Genetic comparison of pathways for aromatic catabolism in the two taxa has revealed some similarities and marked differences. In general, isofunctional enzymes from the two taxa have amino acid sequence identity of roughly 50%. Differences are evident at the DNA level in that the *Pseudomonas* genes characteristically have a G+C content above 58% (32) whereas the *Acinetobacter* genes, with a few notable exceptions, have a G+C content below 46% (42). Numerous rearrangements accompanied the divergence of the respective genes, yet transposition did not always lead to their scattering in the chromosome of their hosts. Apparent selection for grouping of genes with related physiological function is evident in the *Acinetobacter pca-qui-pob* supraoperonic cluster (Fig. 1) in a chromosomal segment containing more than 20 kb of DNA (11, 12, 16, 17, 26, 42). Recent investigations have demonstrated tight linkage

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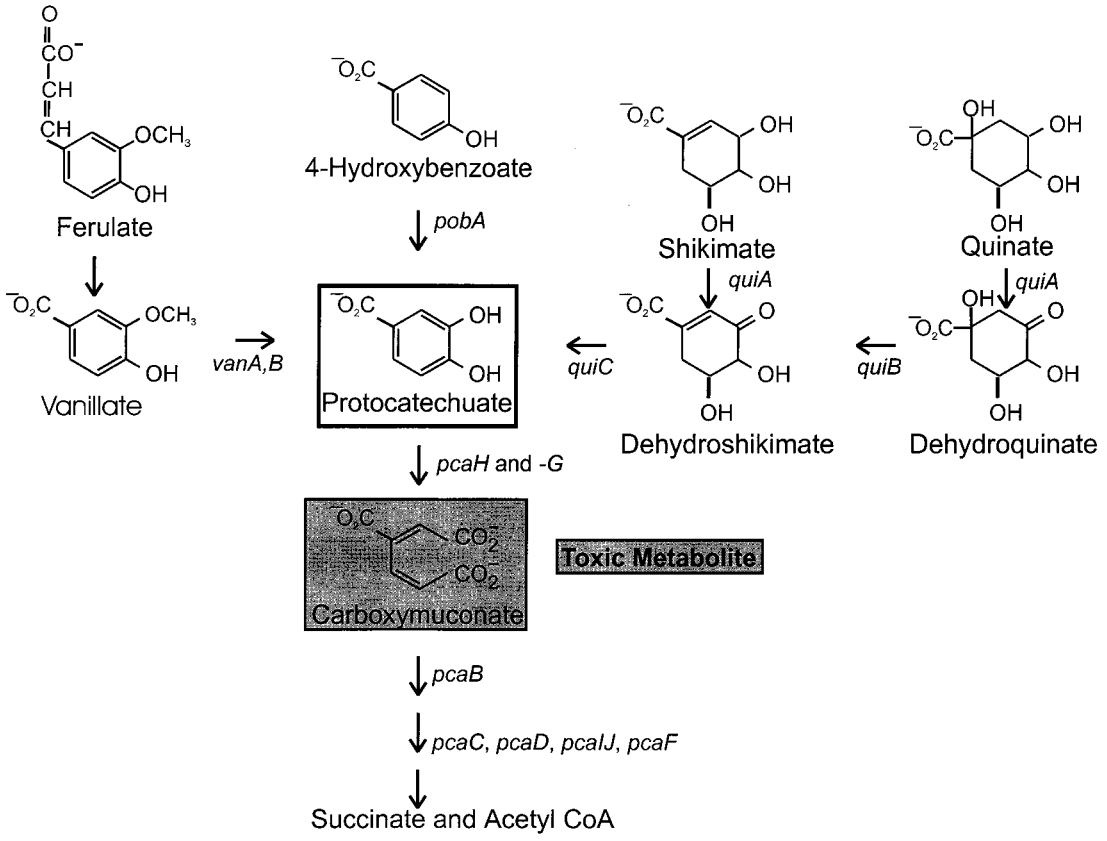

FIG. 1. Vanillate and many other plant products are metabolized through protocatechuate. The *pob*, *qui*, and *pca* genes are clustered in the *Acinetobacter* chromosome. The open box indicates protocatechuate, and the shaded box indicates carboxymuconate, which is produced by the action of protocatechuate 3,4-dioxygenase on protocatechuate. Arrows indicate metabolic reactions and are accompanied by the designations of genes encoding the enzymes that catalyze the reactions. Metabolic accumulation of carboxymuconate in strain ADP230, defective in *pcaB*, prevents the growth of cells in the presence of substrates that can be metabolized to carboxymuconate.

to *pobA* of a gene encoding a CoA-ligase required for catabolism of ferulate and its structural analogs, caffeate and coumarate (61). An objective of the present study was to determine if *vanA* and *vanB* were linked to the *pca-qui-pob* gene cluster.

Earlier investigations demonstrated that vanillate and protocatechuate (Fig. 1) are formed during the metabolism of ferulate by *Acinetobacter calcoaceticus* DSM586 (10). The conclusion that the vanillate pathway was the sole mechanism for ferulate utilization in *Acinetobacter* would be supported by the demonstration that mutations blocking vanillate demethylase prevented growth on ferulate. An organism well suited for such genetic analysis is *Acinetobacter* strain ADP1. This organism, formerly assigned to the species *A. calcoaceticus* but now recognized to be a representative of a separate taxon within *Acinetobacter* (3, 4, 15), is highly competent for natural transformation (36, 37). Furthermore, the physiological properties of this strain allow the design of a strategy for selection of mutants blocked in ferulate metabolism (Fig. 1).

Such a selection procedure was first used to survey the properties of spontaneous mutants blocked in *pcaH* and *pcaG*, structural genes for protocatechuate 3,4-dioxygenase. Among 94 independently selected strains, 4 contained a newly discovered insertion sequence, IS*1236*, within *pcaH* (25). Similar selection for strains blocked in *p*-hydroxybenzoate metabolism (Fig. 1) yielded strains with defects in either *pobA*, the structural gene for *p*-hydroxybenzoate hydroxylase, or *pobR*, which encodes the transcriptional activator of *pobA* (11, 13). Spontaneous mutations blocking *pobR* are caused predominantly by insertion of IS*1236* at different locations throughout the gene (24).

In this report we describe the isolation of spontaneous *vanA* and *vanB* mutants and demonstrate that the genes for vanillate demethylase are essential for the growth of *Acinetobacter* with ferulate. A significant fraction of the *vanA* and *vanB* mutations were caused by IS*1236*, which also was shown to contribute to the structure of a newly discovered composite transposon, Tn*5613*, which can inactivate the *van* genes. Unlike other known genes associated with protocatechuate metabolism, *vanA* and *vanB* occupy a location separate from the *pca-qui-pob* cluster in the *Acinetobacter* chromosome.

MATERIALS AND METHODS

Bacterial strains and plasmids. Properties of bacterial strains used in this investigation are summarized in Table 1, and relevant properties of plasmids are presented in Fig. 2. *Escherichia coli* cultures were grown at 37°C in Luria-Bertani (LB) medium (60). *Acinetobacter* cultures were grown at 37°C in basal medium supplemented with carbon sources as indicated in the text.

Isolation of mutant strains. Selection for loss of *van* functions was imposed by demanding growth of strain ADP230, a mutant that accumulates the toxic intermediate carboxymuconate when exposed to vanillate, on plates containing succinate as a growth substrate and either vanillate or ferulate as a source of the toxic metabolite (Fig. 1). After genetic restoration of the ability to metabolize carboxymuconate, natural transformation was used to map the mutations blocking vanillate metabolism (24, 30, 42).

Mutagenesis with mini-Tn*10* (34) was achieved by mixing 4×10^7 *E. coli*

TABLE 1. Bacterial strains used in this investigation

Strain	Parental strain	Genotype	Nature of mutation	Selection	Source or reference
ADP1	BD413	Wild type			36
ADP230	ADP1	*pcaΔBDK1*	Engineered deletion		30
ADP642	ADP230	*pcaΔBDK1 ΔvanAB643*	Spontaneous deletion	Resistance to ferulate	This investigation
ADP643	ADP642	*ΔvanAB643*		Growth with protocatechuate	This investigation
ADP644	ADP230	*pcaΔBDK1 vanA645*	Spontaneous insertion	Resistance to ferulate	This investigation
ADP645	ADP644	*vanA645*		Growth with protocatechuate	This investigation
ADP646	ADP230	*pcaΔBDK1 vanB647*::Tn*5613*	Spontaneous insertion	Resistance to ferulate	This investigation
ADP647	ADP646	*vanB647*::Tn*5613*		Growth with protocatechuate	This investigation
ADP648	ADP230	*pcaΔBDK1 vanB649*	Spontaneous bp substitution	Resistance to ferulate	This investigation
ADP649	ADP648	*vanB649*		Growth with protocatechuate	This investigation
ADP650	ADP230	*pcaΔBDK1 ΔvanAB651*	Spontaneous deletion	Resistance to ferulate	This investigation
ADP651	ADP650	*ΔvanAB651*		Growth with protocatechuate	This investigation
ADP652	ADP230	*pcaΔBDK1 ΔvanA653*	Spontaneous 13-bp deletion	Resistance to ferulate	This investigation
ADP653	ADP652	*ΔvanA653*		Growth with protocatechuate	This investigation
ADP654	ADP230	*pcaΔBDK1 ΔvanAB655*	Spontaneous deletion	Resistance to ferulate	This investigation
ADP655	ADP654	*ΔvanAB655*		Growth with protocatechuate	This investigation
ADP656	ADP230	*pcaΔBDK1 vanB657*	Spontaneous insertion	Resistance to ferulate	This investigation
ADP657	ADP656	*vanB657*		Growth with protocatechuate	This investigation
ADP672	ADP230	*pcaΔBDK1 vanA673*::Tn*10*[a]	Mini-Tn*10*	Resistance to both vanillate and kanamycin	This investigation
ADP673	ADP672	*vanA673*::Tn*10*[a]		Growth with protocatechuate; loss of Kanamycin resistance	This investigation
ADP674	ADP230	*pcaΔBDK1 vanA675*::Tn*10*[a] *vanB675*::Tn*5613*	Mini-Tn*10*; spontaneous mutation	Resistance to both vanillate and kanamycin	This investigation
ADP675[b]	ADP672	*vanA675*::Tn*10*[a] *vanB675*::Tn*5613*		Growth with protocatechuate; resistance to kanamycin	This investigation

[a] The portion of Tn*10* remaining in these strains is the 50-bp segment left by nearly precise excision (58).
[b] This strain retains the mini-Tn*10* kanamycin resistance marker, but it is not in the *van* genes.

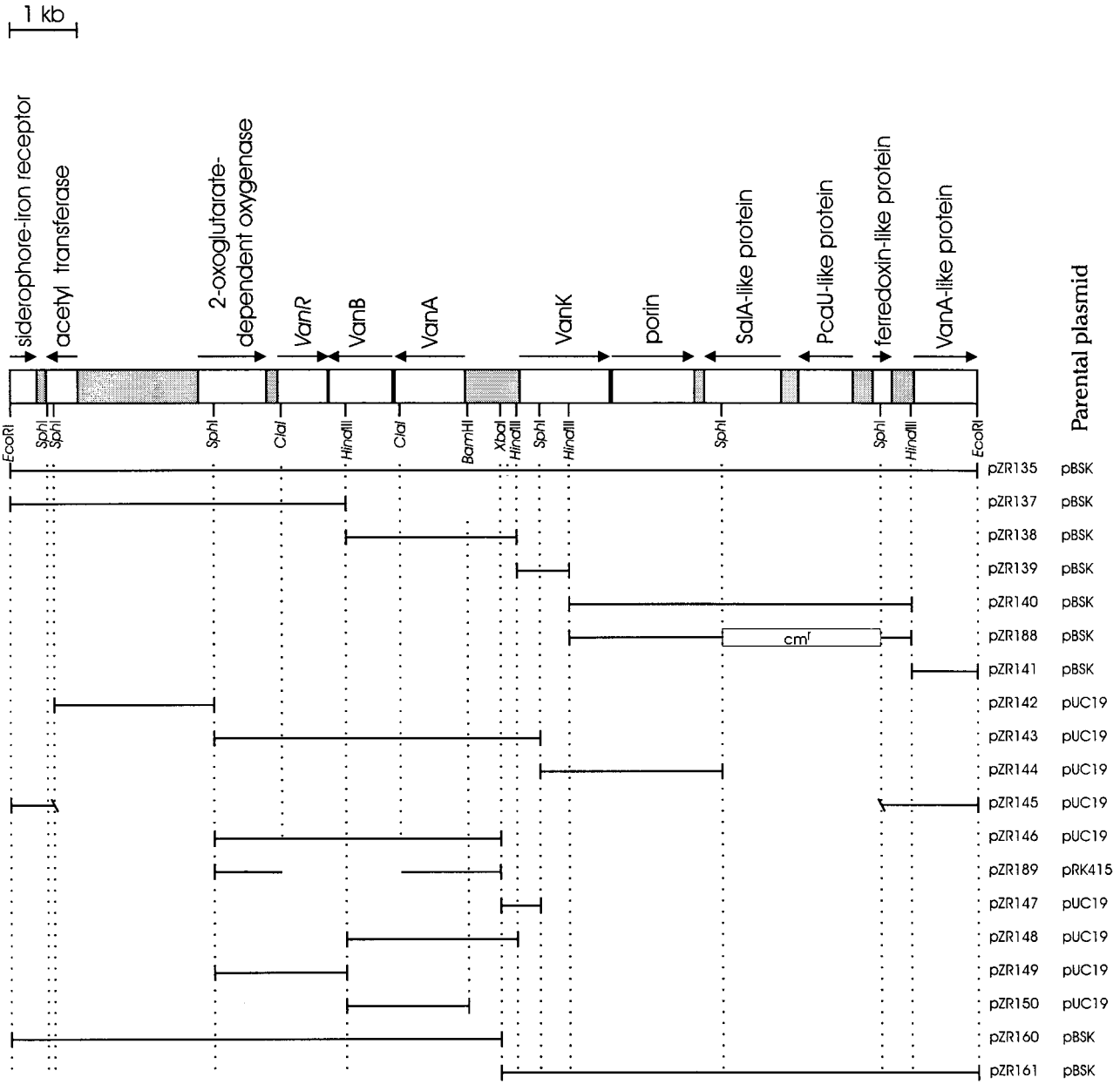

FIG. 2. Plasmids used in this investigation. The DNA in each depicted insert is represented as a horizontal line extending between the restriction sites that mark the limits of the insert. The vectors pRK415 (39), pUC19 (66), and pBSK (Stratagene) have been described previously. All of the inserts were derived from the 14-kb *Eco*RI fragment in pZR135. The interruption in the horizontal line in pZR189 indicates the deletion produced by digestion with *Cla*I before recovery by gap repair of *vanB* chromosomal DNA in this plasmid. At the top of the figure are listed the functions tentatively assigned to open reading frames (indicated as open rectangles) on the basis of amino acid sequences similar to those of known proteins; the arrows indicate the directions of transcription. It must be emphasized that the some of the tentative functions assigned to proteins were shown to be incorrect as part of this investigation: a knockout mutation blocking the expression of the SalA-like protein did not prevent growth on salicylate, and the VanA-like protein did not complement mutations blocking the expression of *vanA*. Not depicted here are four additional *Bam*HI sites (GGATCC) that were detected by sequencing the *vanA-vanK* intergenic region.

SM10λpir(pLOFKm) cells and 10^7 *Acinetobacter* strain ADP230 cells on LB plates containing 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG). After 8 h of incubation, the cells were transferred from these plates and spread on plates containing 10 mM succinate, 2 mM vanillate, and 25 mg of kanamycin per ml. Colonies emerging on this medium were screened for resistance to 2 mM ferulate. During maintenance in the absence of kanamycin, the cell line derived from ADP672 lost resistance to the antibiotic, giving rise to ADP673 (Table 1).

Colonies containing spontaneous mutants were selected by spreading about 10^8 ADP230 cells on plates containing 3 mM ferulate and 10 mM succinate. After single-colony isolation on the same medium, mutant strains were screened for their ability to grow on plates containing 10 mM succinate and 2 mM vanillate. The properties of strains that grew on this medium are summarized in Table 1.

Natural transformation. Recipient *Acinetobacter* cells were grown in a shaker overnight in 5-ml cultures with 10 mM succinate, and succinate was added to an additional concentration of 10 mM the next morning. After 30 min of incubation, 200 μl of the culture was mixed with either cell lysate or plasmid DNA on a plate of selective medium. For transformation prior to selection, 200 μl of the overnight culture was added to a tube containing 5 ml of 10 mM succinate and incubated for 3 h, after which the cells were plated on selective medium.

Manipulation of DNA. Chromosomal DNA and cell lysate were prepared as previously described (25). Plasmid DNA was isolated with the Wizard Miniprep

kit from Promega. Standard techniques of molecular biology were used in plasmid and gene manipulations (60).

Screening by replica plating for *E. coli* clones containing the *Acinetobacter* vanillate demethylase DNA. Chromosomal DNA from wild-type *Acinetobacter* ADP1 was digested with *Eco*RI (New England Biolabs), and the resulting fragments were ligated to pBSK (previously linearized with *Eco*RI). The ligated material was introduced into *E. coli* DH5α by transformation, and the transformants were plated onto LB agar plates containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and IPTG. The resulting *E. coli* colonies were screened for the presence of vanillate demethylase genes by assessment of the ability of plated replicas to transform the mutant ADP675 so that recombinants grew with 2 mM vanillic acid as the sole carbon source (2).

DNA sequencing and sequence analysis. Sequencing of double-stranded plasmid DNA or PCR templates was performed in an ABI 373 automated sequencer (Perkin-Elmer ABI) with T3, T7, m13 universal, and reverse primers and synthetic oligonucleotides. Subclones, nested deletions, and PCR fragments were made to complete the 14-kb sequence of the insert in pZR135. PCR fragments were used to sequence mutant genes. Homology searches were performed with the gapped BLAST database search program (1), and sequence analysis was performed with DNAMAN (Lynnon Biosoft).

Designed deletions. Knockout mutations for open reading frames encoding the SalA-like protein and the PcaU-like protein were created by replacing the internal *Sph*I fragment of pZR140 with DNA encoding chloramphenicol resistance in pCAT19 (21). The insert from the resulting plasmid, pZR188 (Fig. 2), was introduced into wild-type *Acinetobacter* strain ADP1 by transformation followed by selection for chloramphenicol resistance. The resulting mutant, strain ADP1070, was tested for its ability to grow with ferulate, vanillate, or salicylate. To construct a plasmid for recovery of DNA by gap repair (28), the *lacZ*::Kn^r cassette from pKOK6 (41) was inserted into the *Nco*I site of *vanB* in pZR143. Digestion with *Xba*I, using one site from the pZR143 polylinker, then yielded a fragment which was ligated into the broad-host-range vector pRK415. Digestion of this plasmid with *Cla*I released DNA containing all of *vanB*, and the remaining linearized plasmid was used to recover chromosomal DNA containing *vanB647*.

***p*-Toluidine test for metabolic accumulation of protocatechuate.** The color test developed by Parke (55) to screen for protocatechuate accumulation was used to detect formation of the compound from vanillate in *E. coli* cells containing different recombinant plasmids.

PCR amplifications. PCR amplifications were performed with 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min. Special conditions were also used with the amplification of ADP647, ADP657, and ADP645 with primers Seq1 to Seq6: the extension time was 5 min, and the annealing temperature was 45°C.

PCR primers. PCR primers of special importance in efforts to characterize *van* mutants were located in open reading frames as follows: Seq1, 5′ GTAACTCG GAGAGACTGTACGTC 3′, regulatory protein (Fig. 2); VanB22, 5′ GACGT ACAGTCTCTCCGAGTTAC 3′, regulatory protein (Fig. 2); Seq3, 5′ GGGAA GTGTTCAGTCAGGTTGCC 3′, *vanB* (positions 1772 to 1749); VanB11, 5′ CGCCTATTCTCTCCATGGC 3′, *vanB* (positions 1655 to 1673); VanB21, 5′ CGTCAATATGTGAGCCTGCG 3′, beginning of *vanB* (positions 1423 to 1403); VanA2, 5′ CAGGCGTAACTACAATCGACAAC 3′, end of *vanA* (positions 979 to 1003); Seq5, 5′ CAGCCCACCGCCATAACTCCACTCT 3′, *vanA* (positions 631 to 606); Seq6, 5′ CAATAAGTGATAAGGAGTCG 3′, upstream of *vanA* (positions 192 to 214); IS1, 5′ GGCCATTTCTTGGATCTCC 3′, 5′ end of IS*1236* (positions 60 to 78); IS2, 5′ CGGCTGGTTAAGCCCAGAAGC 3′, 3′ end of IS*1236* (positions 154 to 1174).

Nucleotide sequence accession numbers. The nucleotide sequence of the 14-kb *Eco*RI fragment containing *Acinetobacter vanA* and *vanB* appears in the GenBank nucleotide sequence database under accession no. AF009672. The GenBank accession number for the complete Tn*5613* nucleotide sequence is AF091240, and the GenBank accession number for the 2.7-kb nucleotide sequence of the partial open reading frame carried as an insert in pZR153 is AF011339.

RESULTS

Isolation of mutants unable to metabolize either ferulate or vanillate to protocatechuate. To test the possibility that vanillate is an intermediate in the catabolism of ferulate by *Acinetobacter*, strains were selected on the basis of their inability to convert ferulate to carboxymuconate, a toxic intermediate that accumulates and prevents growth in strains containing the *pcaΔBDK1* deletion (Fig. 1), and then were screened for potential blocks in their conversion of vanillate to protocatechuate. After exposure to mini-Tn*10*, ADP230 derivatives that had acquired the transposon were selected by demanding growth in the presence of kanamycin, and colonies that exhibited kanamycin resistance were screened for growth with succinate in the presence of ferulate. Further screening of ferulate-resistant isolates revealed two strains, ADP672 and ADP674, that grew with succinate in the presence of either ferulate or vanillate but failed to grow with succinate in the presence of protocatechuate (Table 1). Replacement of the *pcaΔBDK1* deletion in these strains produced the respective transformants ADP673 and ADP675, which grew in the presence of protocatechuate and, as would be expected for strains blocked in vanillate demethylase, failed to grow in the presence of either ferulate or vanillate (Table 1).

As described below, a genetic remnant of mini-Tn*10* remained in the *van* genes of strains ADP673 and ADP675, but the kanamycin marker in these strains was lost and transposed nearby in the chromosome. It therefore was evident that multiple mutations accompanied mini-Tn*10* inactivation of the *van* genes in these organisms, and in order to rule out the possibility that such transpositions-induced mutations affected additional genes required for vanillate catabolism, spontaneous mutants blocked in conversion of vanillate to protocatechuate were selected. Eight spontaneous vanillate-resistant derivatives of strain ADP230 were independently obtained and shown to be unable to grow in the presence of protocatechuate (Table 1). As would be expected if vanillate demethylase were required for ferulate catabolism, the mutant strains grew in the presence of ferulate. Replacement of *pcaΔBDK1* in the spontaneous mutants gave rise to strains that grew at the expense of protocatechuate and not at the expense of either vanillate or ferulate (Table 1).

Cloning of DNA fragments containing genes for vanillate demethylase. Screening of *E. coli* colonies carrying a library of *Eco*RI fragments of *Acinetobacter* DNA revealed four strains, each of which contained a different recombinant plasmid that transformed *Acinetobacter* strain ADP675 so that it grew on vanillate. Two of the plasmids (pZR135 and pZR136) contained 14-kb inserts in opposite orientations with respect to the plasmid promoter. The other two plasmids (pZR151 and pZR156) contained inserts of 3.3 and 5.5 kb, respectively. As judged by the *p*-toluidine color test (55), *E. coli* DH5α (pZR135) produced a substantial amount of protocatechuate from vanillate, whereas *E. coli* DH5α(pZR136), containing the 14-kb *Eco*RI insert in reverse orientation with respect to the plasmid *lac* promoter, formed a slight amount of protocatechuate and *E. coli* strains containing pZR151 or pZR156 did not form discernible amounts of protocatechuate.

Subcloning and characterization of mutant strains by gene replacement. Plasmid pZR135 and the five *Hin*dIII subclones derived from it (Fig. 2) were tested for their ability to restore wild-type function to the 10 mutant strains blocked in the conversion of vanillate to protocatechuate. The plasmids did not produce a wild-type phenotype when used as donors in transformation for strains ADP643 or ADP655; these strains did yield wild-type recombinants when treated with DNA from the parental strain ADP1. These mutant strains and strain ADP651 did not yield wild-type recombinants when used as the donor in transformation of the other strains blocked in vanillate catabolism. It thus appears that strains ADP643 and ADP655 have undergone deletions extending beyond the limits of the insert in pZR135; the deletion in strain ADP651 eliminates alleles for which *van* mutations were obtained but falls within the confines of the pZR135 insert. The remaining *van* mutants exhibited the ability to be transformed to the wild type by pZR135 and by either of two *Hin*dIII subclones, pZR138 or pZR137 (Fig. 2). This evidence suggested that the *Hin*dIII restriction site between the chromosomal fragments inserted in pZR138 and pZR137 (Fig. 2) lies within the genes required for vanillate catabolism.

Most of the mutant strains that were transformed by one of

the *Hin*dIII subclones appear to have mutations at separate loci as evidenced by growth of wild-type recombinants after crosses between pairs of the strains. Exceptions to this pattern indicated that some independent mutations may have occurred at identical or nearby loci, and subsequent sequencing of the mutant DNA proved that this was the case. Anomalous results were obtained with strain ADP675, which subsequently was shown to have acquired two separate mutations in the *van* region.

Nucleotide sequences of different *Acinetobacter Eco*RI restriction fragments that transform a *van*-deficient strain so that it grows with vanillate. Three different *Eco*RI chromosomal fragments conferred upon strain ADP675 the ability to grow with vanillate. The 5.5-kb insert of pZR156 contained an internal *Eco*RI site. Sequence analysis of the ends of the pZR156 insert revealed DNA corresponding to *benK* and *benD*. The location of restriction sites within the insert confirmed that the cloned genes encoded proteins required for the oxygenative metabolism of benzoate and corresponded to the insert of the previously described plasmids pIB1351 and pIB1352 (49, 50), together with an *Eco*RI fragment internal to *benD*. The 3.3-kb insert of pZR151 also contained an internal *Eco*RI site; a 2.7-kb *Eco*RI subclone in pZR153 remained able to confer upon ADP675 the ability to grow on vanillate. Sequencing of the 2,755-bp insert in pZR153 revealed that it consisted entirely of a large partial open reading frame beginning with sequences encoding tandem repeats of approximately 100 amino acids, similar to the fibronectin type III repeats in bacterial cellulases (29, 45, 46).

The complete 14,358-bp nucleotide sequence of the pZR135 insert revealed 12 open reading frames and two large DNA segments (one of 1,781 bp and one of 811 bp) with no obvious coding functions (Fig. 2). The open reading frames are of general interest because they are components of a 100-kb DNA fragment that has been selected in wild-type *Acinetobacter* yet is frequently lost during maintenance of the organism in the laboratory (27).

Of immediate significance to this investigation were the two open reading frames encoding proteins with amino acid sequences closely resembling those determined for VanA and VanB, proteins that form vanillate demethylase in *Pseudomonas* species (5, 57, 62). The possibility that the *Acinetobacter* open reading frames assigned *vanA* and *vanB* function was enhanced by the fact that DNA from either pZR137 or pZR138 (Fig. 2) replaced mutations in these genes and, as described below, was confirmed by sequencing of mutant genes.

The locations of *Acinetobacter vanA* and *vanB* are indicated in Fig. 2. Also indicated is an apparent regulatory gene, tentatively designated *vanR*, that converges upon *vanB*; the convergently transcribed genes overlap by 21 bp.

To identify regions that might be associated with transcriptional termination, the 14-kb sequence of the *Eco*RI fragment was scanned for inverted repetitions containing complementary sequences exceeding 10 bp. The strongest free energy of association, 10.1 kcal/mol, was exhibited by an 11-bp palindrome separated by 7 bp. The palindrome begins 13 bp downstream from the translational terminus of *vanB* and is followed by the sequence TTTTTT. This potential terminator of the *vanA* and *vanB* transcript is of particular interest because it lies well within the convergently transcribed *vanR*.

The longest palindromic pair proved to be 14 bp long and separated by 401 bp. This inverted repetition and an additional inverted repetition of 12 bp are within the 811-bp *vanK-vanA* intergenic region; all of the repeated sequences contain the internal palindromic sequence TGGATCCA. Further examination showed that GGATCC, the *Bam*HI restriction site, occurs five times in the 811-bp *vanK-vanA* intergenic region and only three times in 70,000 bp of known nucleotide sequence from *Acinetobacter* strain ADP1. A close match to GGATCC is exhibited by the termini of *IS1236*, which are TGATCC and GGATCA. It is possible that palindromes containing the sequence GGATCC served as a class of preferred targets for IS*1236* integration.

Among open reading frames that resemble *Acinetobacter vanA* is the partial open reading frame at one end of the pZR135 insert. Over 171 aligned residues, the protein encoded by this open reading frame has identity of 33% to *Acinetobacter* VanA, less than the sequence identity of 72% for *Acinetobacter* VanA and its closest known homolog, VanA from *Pseudomonas* sp. strain HR199 (57).

Two neighboring open reading frames in pZR135 closely resemble open reading frames with known functions in aromatic catabolism. One of these, designated the gene for a SalA-like protein in Fig. 2, encodes a protein with amino acid sequence identity of 30% to the known *P. putida* salicylate monooxygenase (68). The other open reading frame, whose product is designated PcaU-like protein in Fig. 2, encodes a product that resembles *Rhodococcus opacus* PcaR (30% sequence identity [18]) and *Acinetobacter* PcaU (27% sequence identity), the transcriptional activator of genes for protocatechuate catabolism (26). The ferredoxin-like protein (Fig. 2) most closely resembles (33% amino acid identity over 100 residues) a *Pseudomonas* protein, NagAb, believed to be part of the electron transport chain for both salicylate 5-hydroxylase, which converts salicylate to gentisate, and naphthalene dioxygenase (20).

The functions of the *salA*-like and *pcaU*-like genes were explored by introduction of a deletion mutation removing 410 bp from the transcriptional terminus of the 813-bp *pcaU*-like gene and 886 bp from the beginning of the *salA*-like gene. Strains containing this mutation were unimpaired in their catabolism of salicylate, vanillate, and protocatechuate as judged by growth on plates; therefore, the altered genes appear to be associated with other functions.

Two genes that may form a transcriptional unit in pZR135 appear to be associated with transport. One of these, designated the gene for VanK in Fig. 2, encodes a product with amino acid sequence with 31% identity to *Acinetobacter* PcaK, a member of a family of proteins known to transport *p*-hydroxybenzoate and protocatechuate in bacteria (7, 31, 35, 42, 52, 63). The other transport related open reading frame, indicated as a gene with outer membrane protein (OMP)-like function in Fig. 2, encodes a product with 32% sequence identity to its closest known homolog, *P. putida* PhaK, which is required for growth of the organism on phenylacetate (53). The putative *Acinetobacter* porin also exhibits close sequence similarity to four gene products assigned OMP functions in *Pseudomonas aeruginosa* (65, 67); comparison of the products of the *Acinetobacter* OMP-like gene with the *Pseudomonas* genes revealed amino acid sequence identities ranging between 25 and 31%.

Upstream of the regulatory gene that overlaps with *vanB* (Fig. 2) is an ORF encoding a protein with up to 27% identity to several members of the 2-oxoglutarate-dependent oxygenase family in a variety of plants. Because two members are multifunctional gibberellin oxidases that can convert the aldehyde of this plant hormone to its acid (43), this raises the possibility that the *Acinetobacter* enzyme catalyzes the conversion of vanillin to vanillate. In *Pseudomonas* sp. strain HR199, this function is performed by an NAD-dependent dehydrogenase and its gene is near *vanAB* on the chromosome (57).

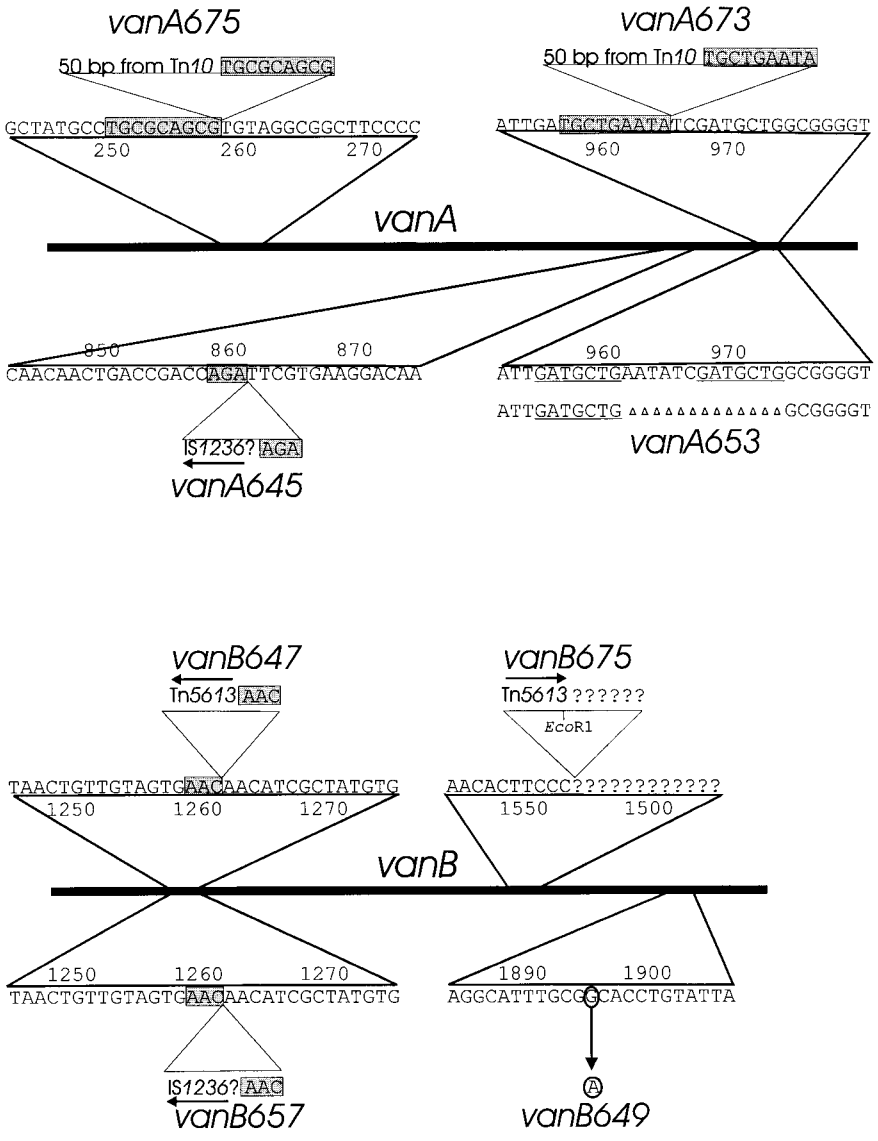

FIG. 3. Characterized mutations in *vanA* and *vanB*. Linear bars represent *vanA* and *vanB*; expanded portions of the sequence depict the locations of mutations. Shaded rectangles indicate direct repetitions of chromosomal sequence flanking sites of insertion for mutations caused by insertion sequences or transposons. Horizontal arrows show the direction of transcription of the open reading frames within IS*1236*. Underlining marks the 7-bp direct chromosomal sequence repetition that appears to have directed the 13-bp *vanA653* deletion. Characterization of DNA upstream from *vanB674* was made possible by cloning of an *Eco*RI fragment containing DNA extending into the *Eco*RI site of Tn*5613*. Properties of DNA downstream from this site in *vanB675* remains unknown.

Transcribed divergently from this oxygenase gene, cloned in pZR135 and separated by over 1,700 bp of DNA without any obvious coding potential, is an ORF for a protein with up to 31% identity (48 of 152 aligned residues) to various yeast hypothetical acetyltransferases. This protein is less closely related to an *N*-acetyltransferase that provides resistance to the antibiotic streptothricin in diverse bacteria. Lastly, on the end of the pZR135 insert and transcribed in the opposite direction to the putative acetyltransferase gene is the end of a gene encoding a protein with 23 to 33% identity to the corresponding segment of various iron-scavenging outer membrane receptors including the *E. coli* FhuA ferrichrome receptor, the *P. aeruginosa* FptA iron-pyochelin receptor, and the *Vibrio anguillarum* FatA iron-anguibactin receptor. Ligands for the last two receptors are structurally related (9, 56, 64) to other pathogenic bacterial siderophores including yersiniabactin (56) from various *Yersinia* species and acinetobactin (14, 64) from *Acinetobacter baumanii*.

Nucleotide sequences of *vanA* and *vanB* genes containing spontaneous mutations. The primers Seq1 and Seq6 were used to amplify the chromosomal DNA containing *vanA* and *vanB* from the *Acinetobacter* chromosome, and internal primers (Seq2, Seq3, and Seq5) were used to sequence mutations in these genes. As expected, the PCR did not yield amplified fragments with the deletion mutations Δ*vanAB643*, Δ*vanAB651*, and Δ*vanAB655*. Sequencing revealed that Δ*vanA653* is a 13-bp deletion removing 7 bp of directly repeated nucleotide sequence (Fig. 3), suggesting that the mutation was directed in part by hybridization between slipped DNA strands (Fig. 4).

Only one of the eight selected spontaneous mutations is a base substitution: the *vanB649* mutation contains G-to-A nucleotide substitution causing the amino acid substitution

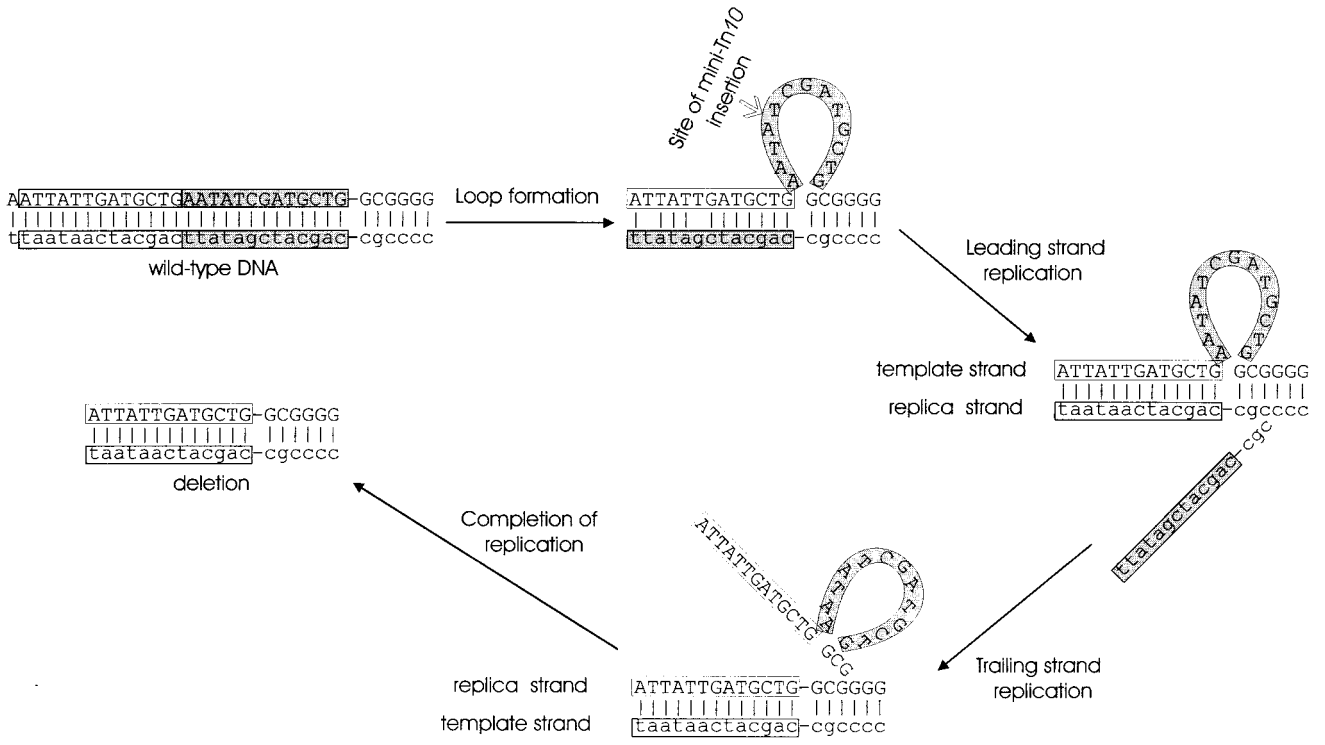

FIG. 4. Possible contributions of DNA strand slippage to mutations in *vanA*. The 11-bp deletion in *vanA653* removed a 7-bp direct sequence repetition, suggesting that mispairing between slipped strands may have misaligned DNA so that the deletion took place during replication. The single-stranded loop caused by mispairing may have been a target for mini-Tn*10* insertion, resulting in *vanA675*.

G269D in VanB (Fig. 3). The substituted amino acid is underlined in the peptide sequence CEQGICGTCITR, which is believed to be associated with iron-sulfur binding and is completely conserved in four different *Pseudomonas* isolates. The glycyl residue is conserved in enzymes as distantly related as pthalate dioxygenase reductase (8), toluenesulfonate methylmonooxygenase reductase (38), and 3-chlorobenzoate-3,4-dioxygenase reductase (47); therefore, replacement of the flexible neutral amino acid residue by the charged aspartyl residue would be expected to be highly disruptive to the protein structure.

Unexpectedly, chromosomal DNA containing any of the remaining three spontaneous mutations, *vanA645*, *vanB647*, or *vanB657*, could not be amplified by PCR, prompting the hypothesis that these mutations contained long DNA inserts. Since IS*1236* is a known cause of insertion mutations in *Acinetobacter* (24), the primers Is1 and Is2, complementary to nucleotide sequences near the left and right termini, respectively, of IS*1236* (24), were paired with primers from *vanA* and *vanB* to determine if the insertion sequence could be located within the mutant genes. The results of this analysis and subsequent nucleotide sequencing demonstrated the contribution of IS*1236* to all three mutations (Fig. 3).

Insertion of a single copy of the 1.2-kb IS*1236*, however, could not account for the three negative PCR results. Therefore, gap repair (28) was tried with the two of the mutants by using a plasmid in which digestion with *Cla*I had removed *vanB* DNA (Fig. 2). An 8-kb DNA insert containing *vanB647* was recovered from the chromosome of ADP647. Sequencing of this fragment revealed a 3-bp direct repeat of *vanB* DNA (Fig. 3) flanking a previously undescribed 3,552-bp composite transposon designated Tn*5613*. At the ends of the transposon are identical copies of IS*1236* in the same orientation. Between these and in the opposite orientation is a 546-bp open reading frame. The G+C content of the open reading frame is 36%, somewhat less than the 39% G+C content of the entire Tn*5613*, and the translation product of the open reading frame shows no obvious similarity to any of the amino acid sequences reported in GenBank.

Procedures used to recover *vanB647* by gap repair did not yield a product when applied to strains containing *vanB657*. Sequencing of PCR-amplified DNA flanking IS*1236* in *vanB657* demonstrated that at least one copy of the IS element is inserted in the same manner as Tn*5613* in *vanB647* (Fig. 3), but PCR with one primer complementary to the open reading frame within Tn*5613* gave no indication of corresponding DNA in *vanB657*. Furthermore, PCRs with primers designed to amplify between the two IS elements of Tn*5613* in strains with *vanB657* or *vanB645* also failed to yield a product (in contrast to results with the other six spontaneous mutants), suggesting that the transposon was not intact in these two strains. The complete nature of the *vanB657* and *vanB645* mutations remains a mystery.

Nucleotide sequences of *vanA* and *vanB* genes containing mutations caused by mini-Tn*10*. Mini-Tn*10* proved to be unstable in the two mutants that emerged after selection for the kanamycin resistance marker carried by this transposon. The marker was lost in the cell line that gave rise to the kanamycin-sensitive strain ADP673, which did not grow with vanillate. Mapping of *vanA673* in this strain suggested that it was extremely close to the 13-bp deletion *van653* (Fig. 3), raising the possibility that the DNA strand slippage giving rise to this mutation influences the behavior of the transposon at the same location (Fig. 4). As indicated in Fig. 3, the departed mini-

Tn*10* left behind a 50-bp signature sequence of nearly precise excision (58), and this sequence is flanked by 9-bp direct repeats (40) of chromosomal nucleotide sequence (Fig. 3).

Multiple mutations emerged in strain ADP675 after its treatment with mini-Tn*10* followed by selection for resistance to the intracellular accumulation of β-carboxymuconate. The mutant strain retained its resistance to kanamycin. The gene conferring this trait was recovered from an *Eco*RI library from the mutant strain and proved to be in a DNA fragment containing a portion of the *van* genes. One end of this *Eco*RI restriction fragment corresponded to the site within a portion of the putative *vanA*-like gene in pZR135 (Fig. 2), and the other *Eco*RI site was within Tn*5613*. Sequence analysis demonstrated that the kanamycin resistance determinant was not in *vanA* or *vanB*. Evidence for its temporary insertion was revealed by *vanA675*, which contains the 50 bp sequence corresponding to nearly precise excision flanked by 9-bp chromosomal repeats (40, 58). Strain ADP675 also contained the portion of Tn*5613* extending to its internal *Eco*RI site in *vanB675* (Fig. 3). This was demonstrated by sequencing from Tn*5613* into DNA upstream from *vanB* (Fig. 3). PCR primers downstream from *vanB675* did not yield an amplified product with chromosomal DNA from ADP675, and so it seemed likely that a portion of this DNA was deleted downstream from the site of Tn*5613* insertion in the mutant strain.

DISCUSSION

***vanA* and *vanB*, essential for metabolism of both ferulate and vanillate, are separated from the *pca-qui-pob* gene cluster in the *Acinetobacter* chromosome.** At the outset of this investigation, it was not clear whether vanillate was an obligatory intermediate in the utilization of ferulate by *Acinetobacter*. Essential participation of vanillate as a catabolite is demonstrated by the fact that spontaneous *Acinetobacter* mutations blocking *vanA* and *vanB* prevent growth with ferulate, and this growth property is restored by exposure of the mutant cells to DNA containing the wild-type genes. Such a conclusion could not be drawn on the basis of sequence evidence alone. The 14-kb DNA fragment containing *vanA* and *vanB* also contains an open reading frame resembling *vanA* in sequence but apparently not involved in growth with vanillate. Also within this DNA fragment is a gene that, on the basis of sequence similarity, appears to encode salicylate hydroxylase. However, inactivation of this open reading frame does not prevent growth with salicylate, and so the function of this gene awaits elucidation.

The precise locations of *vanA* and *vanB* are unknown, but the genes appear to be lacking in organisms that have undergone the spontaneous deletion eliminating a 100-kb DNA fragment from the *Acinetobacter* ADP1 chromosome (27). Such strains grow with protocatechuate but not with vanillate and do not reveal DNA fragments corresponding to portions of *vanA* or *vanB* on PCR with primers that do produce such fragments after amplification of wild-type chromosomal DNA. The 100-kb deletion was fortuitously discovered during mapping of the *Acinetobacter* chromosome and occurs in a region that appears to contain multiple copies of IS*1236* (24, 27), perhaps reflecting in part the presence of Tn*5613*. If the *van* genes do lie within the deleted region, then preferential localized transposition of Tn*5613* may explain why this transposon was not detected previously among spontaneous mutations in *pob* or *pca* genes. The discovery of Tn*5613* adds a new genetic marker that may facilitate the typing of *Acinetobacter* strains and rapid identification of the strains most likely to be associated with hospital infection (3, 4, 15). The identity of Tn*5613* as a transposon associated primarily with *Acinetobacter* and not with *Pseudomonas* is affirmed by the G+C content of 39% for the transposon as a whole and 36% for the internal open reading frame.

The 100 kb of spontaneously deleted DNA and by implication the *van* region are distant from the 20-kb *pca-qui-pob* gene cluster in the *Acinetobacter* chromosome (27). This makes *vanA* and *vanB* the only genes associated with the metabolic activities summarized in Fig. 1 that are separate from the cluster. It is possible that the locations of *vanA* and *vanB* are related to their evident instability. Loss of the genes would cause ferulate to be converted to vanillate, while coumarate and caffeate, which are related to ferulate in both structure and nutritional source, would be metabolized completely through protocatechuate. Thus, the existence of spontaneous *van*-deficient strains in natural *Acinetobacter* populations might allow the production of vanillate from ferulate as a chemical signal between plants and bacteria.

Spontaneous mutations caused by Tn*5613*. Insertions of IS*1236* at different positions in *Acinetobacter pobR* cause most of the spontaneous mutations in this gene and are accompanied by a target site duplication of 3 bp (24). Insertion of IS*1236* into *pcaH* is accompanied by less precise duplications, and such insertions appear to cause only about 10% of the spontaneous mutations in *pcaH* and *pcaG* (25). In this study, the newly discovered transposon Tn*5613*, which includes two copies of IS*1236*, was found to be a significant contributor to spontaneous mutation in *vanA* and *vanB* and to have generated a 3-bp duplication upon insertion in *vanB647* (Fig. 3). The mutations *vanA645* and *vanB657* appear to be more complex but include at least one copy of IS*1236* as well as a 3-bp target site duplication. Insertion of a different transposon in these two strains, composed of IS*1236* elements including one from Tn*5613*, is consistent with the inability to PCR amplify across the mutation together with the inability to detect by PCR an intact Tn*5613* element.

Contrasting arrangement in the *Pseudomonas* chromosome of genes flanking *vanAB*. The overlap of the 3′ ends of *vanB* and the convergently transcribed putative regulatory gene *vanR*, an arrangement not found previously in ADP1 for genes involved in aromatic catabolism, prompted an analysis of DNA including *vanA* and *vanB* in other organisms. DNA for a VanR homolog, a regulatory protein in the GntR family (33), was identified in two pseudomonads, but, unexpectedly, in *P. putida* WCS358 (62) it was convergently transcribed from *vanB*, as in ADP1, whereas in *Pseudomonas* sp. strain HR199 (57) it was divergently transcribed from *vanA* (Fig. 5). Due either to mutation or sequence data ambiguity, a frameshift is required in both *Pseudomonas* sequences to maximize the amino acid alignment with their putative ADP1 homolog. Furthermore, the *P. putida* WCS358 locus corresponding to *vanK* in ADP1 appears to be occupied by a gene for the periplasmic ATP-binding protein component of an ABC-type transport system (Fig. 5). The VanK amino acid sequence identifies it as a member of the major facilitator superfamily of transport proteins, structurally distinct from ATP-binding cassette transporters (54); therefore, significantly different classes of protein appear to have been called upon during evolution of the transport capacity associated with the *vanAB* region in different soil bacteria. Based on preliminary sequence information from the *Pseudomonas* Genome Project, *P. aeruginosa* PAO1 has a *vanAB* region similar in organization to that of *Acinetobacter* sp. strain ADP1 (Fig. 5). The presence of this unusual gene organization in the two genera cannot be attributed to recent horizontal transfer between *Pseudomonas* and *Acinetobacter*,

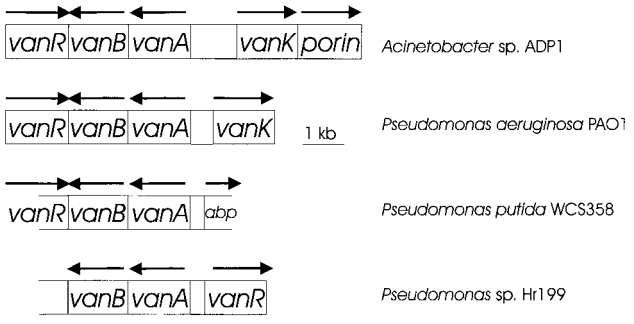

FIG. 5. Comparative organization of the *vanAB* chromosomal region. From top to bottom in the figure, the percent amino acid identities to the corresponding proteins in *Acinetobacter* sp. strain ADP1, followed in parentheses by the numbers of aligned residues, are 69% (340), 69% (346), and 72% (340) for VanA; 46% (314), 47% (316), and 48% (316) for VanB; 61% (163), 57% (26), and 43% (166) for the regulatory protein in the GntR family (VanR); and 50% (415) for VanK. The region labelled *abp* could encode a protein with up to 33% identity over 148 aligned residues with a protein in various bacteria thought to be the periplasmic ATP-binding component of an ATP-binding cassette-type transport system (although this region includes a stop codon the potential open reading frame). Similarly, the size of the regulatory gene shown above assumes a frameshift in all three *Pseudomonas* sequences, due to mutation or sequencing error (and not included in the calculation of amino acid identity for the protein in the bottom two *Pseudomonas* sequences). The *P. aeruginosa* genes are present on one contig from the 15 September 1998 release of data from the *Pseudomonas* Genome Project. Arrows indicate the directions of transcription.

because the respective genes each possess the distinctive G+C contents characteristic of their host chromosome.

Chromosomal linkage of *van* genes to a putative iron-scavenging receptor gene. During mapping of the *Acinetobacter* chromosome, strain ADP1 lost the ability to grow on vanillate, apparently due to the spontaneous deletion of 100 kb of chromosomal DNA including the *van* region. The detection of several copies of IS*1236* in the chromosomal fragment encompassing the deletion in the parental strain (27) and the spontaneous *van* mutants with insertions of Tn*5613*, including *vanB675* with a flanking deletion, is consistent with the spontaneous deletion being mediated in ADP1, directly or indirectly, by transposable elements. Given the linkage of *vanA* and *vanB* to a putative siderophore receptor gene (Fig. 2), the malleability of this region may be analogous to that described for DNA containing genes for iron metabolism in other bacteria (19). In the plague bacterium *Yersinia pestis*, for instance, genes for iron acquisition are located within a pathogenicity island in a 102-kb unstable region of the chromosome that is prone to spontaneous deletion apparently by recombination between flanking IS elements (6, 19).

Having siderophore genes flanked by repetitive DNA sequences can confer several advantages to a bacterium. On the one hand, this arrangement may facilitate tandem amplification of the genetic region (59), with the increased gene dosage leading to increased siderophore generation. During pathogenic interactions, this would be to the detriment of the host, but the siderophores of the fluorescent pseudomonads are associated with improved plant growth (51). On the other hand, spontaneous loss of genes can be advantageous to bacteria in certain niches (44): deletion of genes for ferrisiderophore receptors, for instance, would remove an outer membrane protein commonly used as an entry point for phage or the antibiotics of competing microorganisms (51). In *Acinetobacter*, a frequent additional consequence of deletion of the genetic region containing a putative iron uptake gene would be the loss of *vanA* and *vanB*. Such a loss would modify the bacteria into bioreactors capable of converting ferulate to vanillate.

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