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Substrate Range and Genetic Analysis of *Acinetobacter* Vanillate Demethylase†

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An *Acinetobacter* sp. genetic screen was used to probe structure-function relationships in vanillate demethylase, a two-component monooxygenase. Mutants with null, leaky, and heat-sensitive phenotypes were isolated. Missense mutations tended to be clustered in specific regions, most of which make known contributions to catalytic activity. The vanillate analogs *m*-anisate, *m*-toluate, and 4-hydroxy-3,5-dimethylbenzoate are substrates of the enzyme and weakly inhibit the metabolism of vanillate by wild-type *Acinetobacter* bacteria. PCR mutagenesis of *vanAB*, followed by selection for strains unable to metabolize vanillate, yielded mutant organisms in which vanillate metabolism is more strongly inhibited by the vanillate analogs. Thus, the procedure opens for investigation amino acid residues that may contribute to the binding of either vanillate or its chemical analogs to wild-type and mutant vanillate demethylases. Selection of phenotypic revertants following PCR mutagenesis gave an indication of the extent to which amino acid substitutions can be tolerated at specified positions. In some cases, only true reversion to the original amino acid was observed. In other examples, a range of amino acid substitutions was tolerated. In one instance, phenotypic reversion failed to produce a protein with the original wild-type sequence. In this example, constraints favoring certain nucleotide substitutions appear to be imposed at the DNA level.

Vanillate demethylase is a two-component enzyme classified as a IA oxygenase (25, 28). It comprises a reductase containing both a flavin and a [2Fe-2S] redox center and an oxygenase containing, in addition to a substrate-binding site, an iron-binding site and a Rieske-type [2Fe-2S] cluster. Little is known about how structure influences function in vanillate demethylase. Demethylases involved in the metabolism of *p*-anisate in *Pseudomonas putida* (1) and vanillate in *P. testosteroni* (3, 35) and *P. fluorescens* (5) are known to be air sensitive and unstable. The vanillate demethylases from *P. testosteroni* and *P. fluorescens* are mixed-function oxygenases and have a wide substrate specificity: *m*-anisate, *p*-anisate, *m*-toluate, 3,4,5-trimethoxybenzoate, and 3,4-dimethoxybenzoate were oxidized by vanillate-induced cells (5, 36). As described here, the *Acinetobacter* vanillate demethylase also possesses a broad substrate range.

Inferences can be drawn about the mechanism of vanillate demethylase from results obtained with the evolutionarily related enzyme phthalate dioxygenase (6). In this enzyme, electrons for hydroxylation flow from NADH to flavin mononucleotide to [2Fe-2S] in the reductase and from the Rieske-type [2Fe-2S] center to the Fe^{2+} site in the oxygenase, where oxygen binding and hydroxylation occur (9, 10, 33, 40). As recently shown for the naphthalene dioxygenase, another member of this group of aromatic dioxygenases, Fe1 of the Rieske [2Fe-2S] center is coordinated by two cysteinyl residues and Fe2 is coordinated by two histidyl residues (14, 15, 18). The iron atom at the active site is coordinated by two histidyl residues and one aspartyl residue (18). Aspartate 205 in the catalytic domain of this enzyme has been shown to be essential for activity (31). The C-terminal regions of the α subunit of the oxygenase component of 2-nitrotoluene 2,3-dioxygenase (30) and biphenyl dioxygenase (26) were shown to be responsible for substrate specificity.

In *Acinetobacter* strain ADP1 (37) and in different *Pseudomonas* strains (2, 34, 36), protocatechuate formed by the demethylase undergoes further oxygenative metabolism to carboxymuconate. As shown in Fig. 1, *Acinetobacter* mutants blocked in carboxymuconate metabolism do not grow in the presence of either vanillate or protocatechuate, thus creating a condition allowing selection of strains carrying secondary mutations blocking expression of either vanillate demethylase (37) or protocatechuate oxygenase (8, 11). Genetic analysis has shown that vanillate demethylase is encoded by contiguous genes, *vanA* for the terminal oxygenase and *vanB* for the dioxygenase reductase. *Acinetobacter* VanA and VanB (37) share amino acid sequence identities of 67 to 77% and 44 to 46% with the respective proteins from *Pseudomonas* spp. (2, 34).

PCR introduces nucleotide substitutions in the amplified DNA segment (4, 19, 22, 39, 41). Resulting amino acid substitutions causing defects in the encoded protein can indicate residues that contribute to protein function. Such analysis is augmented with enzymes like *Acinetobacter* vanillate demethylase because of the ease with which the organism integrates PCR fragments into its chromosome by natural transformation (8, 19–21). Since it is possible to select directly for strains with defects in vanillate demethylase (37), the combination of PCR mutagenesis and natural transformation offers special advantages for genetic analysis. The consequences of mutation can be observed directly at the phenotype level under conditions in which the mutant enzyme limits the rate of growth. Thus, it is possible to distinguish enzymes with temperature-sensitive or leaky properties from those with null mutations (8, 19–21). This is particularly important for analysis of an enzyme like

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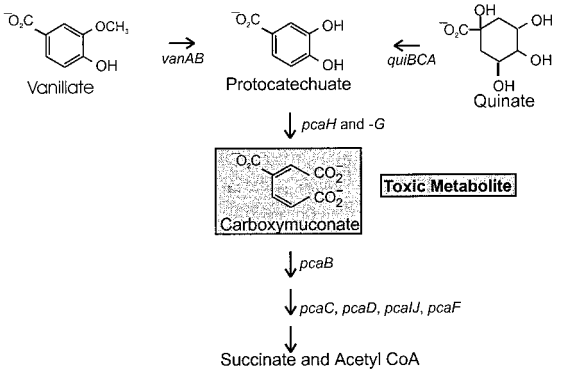

FIG. 1. Selection of strains unable to express either vanillate demethylase or protocatechuate oxygenase. A block in *pcaB* causes accumulation of the toxic metabolite carboxymuconate (12, 37) from vanillate and prevents growth of cells in the presence of this compound. Selection for vanillate-resistant mutants yields strains blocked in either *vanAB*, structural genes for vanillate demethylase (37), or *pcaHG*, structural genes for protocatechuate 3,4-dioxygenase (8, 11). The former class of mutants grows in the presence of vanillate but not in the presence of protocatechuate. Protocatechuate itself is somewhat toxic (7), so quinate was used to select vanillate-defective recombinants in which the *pcaB* mutation has been replaced with wild-type DNA (37). CoA, coenzyme A.

vanillate demethylase, which is not amenable to analysis in cell extracts.

We present here the results of such an analysis of *Acinetobacter* vanillate demethylases with defects caused by amino acid substitution. We also describe mutant demethylases with apparent increased affinity for the substrate analogs 3,4-dimethoxybenzoate, *m*-anisate, *m*-toluate, and 4-hydroxy-3,5-dimethylbenzoate. The results allow identification of amino acid residues likely to be involved in substrate binding and increase understanding of how structure influences function in the enzyme.

MATERIALS AND METHODS

Organisms and culture conditions. The mineral medium described by Juni and Janik (16), supplemented with 10 mM succinate, was routinely used for growth of *Acinetobacter* strains ADP1 and ADP230 in tubes on a shaker or on plates (solidified with 1.8% [wt/vol] agar) at 37°C. Where indicated, vanillate (3 or 1.5 mM) or quinate (3 mM) was used as the carbon and energy source. The structural analogs were added to medium to a final concentration of 3 mM.

Acinetobacter chromosomal DNA containing *vanAB* was cloned for overexpression after PCR amplification with *Taq* polymerase (Quiagen) using primers 5′-ATTGGATCGGTTTCTGGAGCAT-3′ and 5′-GTAGTGAATTCGTAACT CGGAGAG-3′. The latter primer anneals at the end of *vanB* and introduces an *Eco*RI site (underlined) into the primer sequence. The resulting PCR fragment was digested with *Bam*HI and *Eco*RI, gel purified, and ligated into *Bam*HI/*Eco*RI-digested pUC19. Transformants containing the resulting plasmid (pzR9200) in *Escherichia coli* JM109 were isolated by selection for ampicillin resistance and screening for expression of vanillate demethylase in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) induction on plates containing *p*-toluidine (32).

Induction of vanillate demethylase and measurement of vanillate demethylase activity in whole cells. Vanillate demethylase was induced in *Acinetobacter* bacteria by growth of the cells from an overnight inoculum in 10 mM succinate supplemented with either vanillate or one of its chemical analogs at a concentration of 3 mM. After 6 h of incubation, cells were harvested, washed, and resuspended in potassium phosphate buffer (50 mM, pH 7) supplemented with 3 mM vanillate. Samples were taken every 30 min for a total of 3 h, and the remaining vanillate concentration was monitored by high-pressure liquid chromatography.

An overnight Luria-Bertani medium culture of *E. coli* JM109(pzR9200), which expresses the structural genes of vanillate demethylase, was diluted into fresh Luria-Bertani medium (50 ml), and the cultures were grown for about 2 h at 37°C until they achieved turbidity corresponding to an A_{600} of 0.5. IPTG was added to a final concentration of 0.5 mM, and the mixture was incubated for 2 h. At a culture turbidity corresponding to an A_{600} of 1.0, the substrates were added directly to the medium to achieve a final concentration of 5 mM. After 10 to 12 h of incubation, the contents of the flask were centrifuged (10,000 × *g* at 4°C). The supernatant liquids were adjusted to pH 2 to 4 and extracted with ethyl acetate. The extracted material was dried over anhydrous $MgSO_4$.

Analytical methods. Chemical conversions by whole cells were monitored by a reverse-phase high-pressure liquid chromatography system using an LC Pump Model 300 from SSI (Scientific Systems, Inc.), a Shimadzu UV Spectrophotometric detector (model SPD-6A), and a Shimadzu Analyzer (Chromatopac C-R 313). Supernatant liquids from cultures were injected directly into a reverse-phase Nova Pak C_{18} column, eluted with water-methanol (5:1 vol/vol) at a flow rate of 1 ml/min, and monitored at a wavelength of 254 nm. Identification of *m*-hydroxybenzoate, isovanillate, 3-hydroxy-4,5-dimethoxybenzoate, 3-(hydroxymethy)benzoate,3-(hydroxymethyl)-4-methylbenzoate, and 3-(hydroxymethyl)-4-hydroxy-5-methylbenzoate was achieved with a Hewlett-Packard HP 5890 gas chromatograph and an HP 5971A mass spectrometer equipped with an HP5 column. Samples were derivatized prior to gas chromatography-mass spectroscopy analysis as follows: material was methylated with trimethylsilyldiazomethane (13), and any free hydroxyl groups were further protected with bis(trimethylsilyl)acetamide (29). ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra of 3-(hydroxymethyl)-4-methylbenzoate and 3-(hydroxymethyl)-4-hydroxy-5-methylbenzoate were recorded on a Bruker 300-MHz spectrometer at 24°C. Samples for ^{1}H-NMR spectroscopy were purified by flash chromatography with ethyl acetate-hexanes (2:3, vol/vol).

***Acinetobacter* transformation.** *Acinetobacter* bacteria were transformed as previously described (19). A fresh overnight culture, grown in mineral medium with 10 mM succinate as the carbon and energy source, was diluted 25-fold and grown for 2 h at 37°C. About 600 ng of PCR-amplified DNA was added to 500 μl of the fresh culture, which was incubated for 3 h. Dilutions of the transformation mixture were plated directly onto selective medium or onto nonselective medium for determination of viable counts. For selection of spontaneous mutants and as a control, the same protocol was followed but without the addition of DNA.

PCR for transformation-facilitated mutagenesis. *Taq* polymerase (Boehringer Mannheim) was used as indicated by the supplier. Mutagenesis of *vanAB* was performed using the primers Seq1 and Seq2, which were described in a previous study (37). PCR amplifications were carried out with 10 pmol of each primer, 2.5 nmol of each deoxynucleoside triphosphate, 50 to 100 ng of chromosomal template DNA, and 0.5 U of *Taq* polymerase in a total volume of 50 μl. The standard protocol had a total of 35 cycles, with a denaturation step at 94°C, primer annealing at 56°C, and elongation at 72°C. The amplified DNA was used without further purification for transformation of *Acinetobacter* strain ADP230.

Generation and mapping of mutations in *vanAB*. Mutations in the *van* structural genes were selected by the procedure outlined in Fig. 1. After transformation of strain ADP230(Δ*pcaBDK1*) (12) with PCR-amplified *vanAB* DNA, mutant strains were selected on mineral agar medium containing 10 mM succinate supplemented with 3 mM vanillate. The Δ*pcaBDK1* deletion in these mutants was replaced with wild-type DNA by transformation with linearized plasmid pZR3 (12), followed by selection for growth with quinate. The resulting strains were tested at both 22 and 37°C for the ability to utilize vanillate as the sole carbon source either alone or in the presence of 3,4-dimethoxybenzoate, *m*-anisate, *m*-toluate, or 4-hydroxy-3,5-dimethylbenzoate supplied at 3 mM. Mutations in 60 strains were mapped within *vanAB* with PCR-generated DNA fragments of this region (see Fig. 4) as the donors in transformations (37). For these experiments, cells were grown overnight, diluted 25-fold in fresh medium, and grown for another 2 h and 100 μl was plated on basal-medium plates supplemented with 3 mM vanillate; 500 ng to 1 μg of DNA fragment was added to each plate.

Sequence analysis of mutations. The *vanAB* region was amplified by PCR with *Taq* polymerase via the standard procedure for sequence analysis with chromosomal DNA from the mutant strains as the template DNA. PCR-amplified DNA was purified with GeneClean Glassmilk as described by the supplier (Bio 101, Inc.); 200 to 300 ng of the PCR DNA was used as template DNA in cycle sequence reactions with the ABI PRISM dye terminator cycle sequencing kit with Amplitaq DNA polymerase (−FS) as recommended by the supplier (Perkin-Elmer). Cycle sequence products were precipitated with ethanol and sodium acetate (pH 4.8) at −70°C and pelleted in a microcentrifuge at maximum speed. Pellets were washed once with 200 μl of ice-cold 70% (vol/vol) ethanol, air dried for 15 min, and resuspended in a 5:1 (vol/vol) mixture of deionized formamide and 10 mM EDTA (pH 8.0) buffer. DNA fragments were denaturated at 95°C for 2 min prior to electrophoresis on a denaturating 6% polyacrylamide gel in an ABI 373 automated sequencer (Perkin-Elmer ABI) linked to an Apple PowerMac. Sequences were analyzed with the DNA analysis program package DNASTAR (Lasergene).

RESULTS

Activity of VanAB with substrate analogs. The ability of *Acinetobacter* vanillate demethylase to transform different substrate analogs was examined with vanillate-grown *Acinetobacter* cultures. Since such cells might contain enzymes with specificities overlapping that of vanillate demethylase, the survey was repeated with *E. coli* cells in which cloned *Acinetobacter vanAB* had been expressed from the *lac* promoter. Nei-

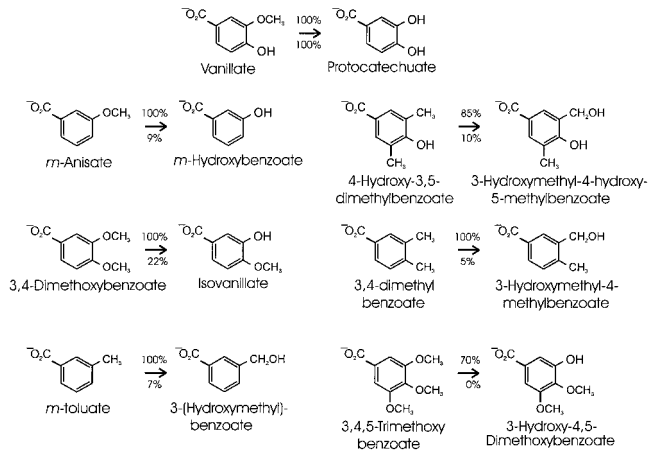

FIG. 2. Metabolic transformations carried out by *Acinetobacter* vanillate demethylase with substrate analogs. None of the analogs supported cell growth. Vanillate demethylase was induced by growing wild-type *Acinetobacter* bacteria with vanillate. The cloned genes for vanillate demethylase were expressed from the *lac* promoter in *E. coli*. Washed cell suspensions at an A_{600} of 1.0 were incubated with vanillate and the depicted chemical analogs. After 10 h, the cells were removed by centrifugation and the relative amounts of the depicted conversions were determined by measurement of the substrate and product concentrations. The respective amounts with vanillate-grown *Acinetobacter* and *E. coli* cells containing vanillate demethylase are shown below and above the arrows. The product of vanillate demethylation in *Acinetobacter* cells was not detected presumably because these cells metabolize the compound completely.

ther the *Acinetobacter* nor the *E. coli* cells revealed detectable activity with *iso*-vanillate, 2,3,4-trimethoxybenzoate, *p*-anisate, *p*-toluate, syringate, 3-methoxy-4-nitrobenzoate, 3-methoxyanisole, *m*-dimethoxybenzene, 3-dimethylaminobenzoate, or *p*-vinylbenzoate. Activities were observed with vanillate and the six substrate analogs depicted in Fig. 2.

Products produced by demethylation of vanillate analogs were identified by mass spectroscopy after methylation and protection of free hydroxyl groups with bis(trimethylsilyl)acetamide. The following values were observed for the *m*/*z* of the fragmentation ion (percentage of base peak and, where mentioned, molecular peak [M^+]): *m*-hydroxybenzoate, 224 (89%, M^+), 209 (100%), 177 (87%), 149 (48%), and 135 (19%); *iso*-vanillate, 254 (27%, M^+), 239 (40%), 224 (100%), 193 (52%), and 165 (13%); 3-hydroxy-4,5-dimethoxybenzoate, 284 (56%, M^+), 269 (82%), 207 (18%), 195 (100%), and 151 (35%); 3-(hydroxymethyl)benzoate, 238 (11%, M^+), 223 (43%), 207 (89%), 177 (40%), 149 (100%), and 133 (21%); 3-(hydroxymethyl)-4-methylbenzoate, 252 (2%, M^+), 237 (19%), 221 (43%), 191 (18%), 163 (77%), 162 (100%), and 131 (50%); 3-(hydroxymethyl)-4-hydroxy-5-methylbenzoate, 340 (9%, M^+), 325 (13%), 309 (11%), 251 (10%), 221 (100%), 207 (10%), and 178 (75%). NMR spectra revealed the following chemical shifts (s indicates singlet, and d indicates doublet) with reference to tetramethylsilane: for 3-(hydroxymethyl)-4-methylbenzoate, 2.4 ppm (3H, s), 4.65 (2H, s), 7.25 (1H, d, J = 6 Hz), 7.8 (1H, d, J = 6 Hz, 8.05 (1H, s); for 3-(hydroxymethyl)-4-hydroxy-5-methylbenzoate, 2.25 ppm (3H, s), 4.75 (2H, s), 7.7 (1H, s), 7.75 (1H, s).

As summarized in Fig. 2, the *E. coli* culture containing cloned *vanAB* exhibited activities substantially higher than those observed with vanillate-grown *Acinetobacter* bacteria. In five of the seven observed transformations, metabolic conversions of 10% or less with the induced *Acinetobacter* cells were greatly exceeded by the *E. coli* cultures, in which the cloned *van* genes caused corresponding conversions exceeding 70%. *E. coli* cultures lacking the cloned genes exhibited no evident activity toward the substrates, so the observed differences can be attributed to elevated expression of the cloned *van* genes. Attempts to determine enzyme activity in cell extracts were not successful, supporting the reports of others about the instability of demethylases (1, 5, 35).

The vanillate analogs that were transformed by vanillate demethylase share the common property of a methoxy or methyl group in a position *meta* to the carboxyl group (Fig. 2). Analogs with a hydroxyl group or only hydrogen in the same position were not transformed. These findings are consistent with the view that the enzyme requires a substituent in the *meta* position for nucleophilic attack. Furthermore, a carboxyl group appears to be essential for substrate binding. The enzyme is able to demethylate one methoxy group or monohydroxylate one methyl group in the *meta* position (Fig. 2).

Three vanillate analogs inhibited the demethylase-catalyzed transformation of vanillate in *Acinetobacter* strain ADP1, consistent with the notion that poorly transformed substrates can competitively inhibit the transformation of good substrates. The rate of vanillate removal by such cells in the presence of 3 mM vanillate was 2 mM/h/mg (dry weight) of cells. Relative rates of 77.5, 40, and 15% were observed in the presence of 1, 2, and 3 mM, respectively, *m*-anisate. At a concentration of 3 mM, 4-hydroxy-3,5-dimethylbenzoate and *m*-toluate produced respective rates of vanillate removal that were 20 and 45% of that observed with vanillate alone. Similar results were obtained with *E. coli* cells containing vanillate demethylase. Growth on plates of wild-type *Acinetobacter* cells with vanillate was inhibited only slightly by the presence of any of the three analogs.

Characterization of PCR-generated *vanAB* mutations. Selection for mutations allowing strain ADP230(Δ*pcaBDK1*) to grow with succinate in the presence of vanillate yielded mutant strains with a frequency 6×10^{-5}. This high frequency is consistent with the previously observed genetic instability of *vanAB*. Even so, transformation of ADP230 with *Taq*-amplified *vanAB* DNA, followed by selection on plates containing both vanillate and succinate, led to a 20-fold increase in the mutation frequency.

After replacement of Δ*pcaBDK1* with wild-type DNA (Fig. 1), the influence of the PCR-generated mutations on growth with vanillate was determined and the mutations were mapped using specified *vanAB* DNA fragments as donors (37). All of the 60 strains analyzed contained mutations mapping in *vanAB*, and the mutant genes in these organisms were sequenced. About half of the sequenced genes contained more than one mutation. Since most of these genes allowed multiple interpretations of how the amino acid sequence influences vanillate demethylase function, they were excluded from further analysis, as were genes containing frameshift mutations. The properties and consequences of the remaining mutations are summarized in Table 1.

Of 34 mutants with defects in *vanAB*, 14 contain stop codons (Table 1). Two of the *vanA* missense mutants are heat sensitive, growing at 22°C but not at 37°C. The growth properties of strain ADP9204 showed that the amino acid substitution W217R rendered the protein unable to support growth at 37°C, but the stability of the protein at the elevated temperature was unknown. This question was addressed by the temperature shift experiment whose results are presented in Fig. 3. When strain ADP9204 was exposed to vanillate at 22°C, the compound was removed after a lag of about 8 h (Fig. 3). A shift in the culture temperature to 37°C stopped the removal of vanillate, and vanillate removal commenced promptly after the culture temperature was restored to 22°C. Thus, the enzyme is

TABLE 1. Nucleotide substitutions that alter translation of *vanA* or *vanB*

Strain	Gene designation	Nucleotide substitution	Amino acid substitution or stop codon created	Phenotype
ADP9200	*vanA9200*	A917C	Q306P	Leaky, increased affinity for inhibitors
ADP9201	*vanA9203*	A914G	Q305R	Leaky, increased affinity for inhibitors
	vanA9199	G670A	A224T	
ADP9202	*vanA9202*	T808A	W270R	Leaky
ADP9203	*vanA9203*	A914G	Q305R	Leaky
ADP9204[a]	*vanA9204*	T649A	W217R	Heat sensitive, increased affinity for inhibitors
ADP9206	*vanA9206*	T437C	L146P	Heat sensitive
	vanA9198	T510C	Silent	
ADP9207	*vanA9207*	T660G	C220W	Null
ADP9208	*vanA9208*	A182T	D61G	Null
ADP9209	*vanA9209*	A448C	N150H	Null
ADP9210	*vanA9210*	T683A	V228D	Null
ADP9211	*vanA9211*	T595C	W199R	Null
ADP9212	*vanA9212*	T452A	L151Q	Null
ADP9213	*vanA9213*	A556C	T186P	Null
ADP9214	*vanA9214*	T658C	C220R	Null
ADP9215	*vanA9215*	A448G	N150D	Null
ADP9216	*vanA9216*	A467G	H156R	Null
ADP9221	*vanA9221*	C485A	S162-TAG stop	Null
ADP9222	*vanA9222*	C982T	Q328-TAA stop	Null
ADP9223	*vanA9223*	T161A	L54-TAG stop	Leaky
ADP9224	*vanA9224*	A502T	K168-TAA stop	Null
ADP9226	*vanA9226*	T288G	Y96-TAG stop	Leaky
ADP9227	*vanA9227*	G393A	W131-TGA stop	Null
ADP9228	*vanA9228*	A740T	K246-TAA stop	Null
ADP9229	*vanA9229*	T521A	L174-TAG stop	Null
ADP9231	*vanA9231*	G809A	W270-TAG stop	Null
ADP9237	*vanB9237*	T103C	S35P	Leaky, increased affinity for inhibitors
ADP9238	*vanB9238*	A337T	I116F	Leaky
	vanA9197	T981C	Silent	
ADP9239	*VanB9239*	T820C	C274R	Null
ADP9240	*vanB9240*	T796A	C266S	Null
ADP9247	*vanB9247*	A312T	K105-TAA stop	Null
ADP9248	*vanB9248*	T171A	C57-TGA stop	Null
ADP9249	*vanB9249*	A28T	K10-TAA stop	Null
ADP9250	*vanB9250*	C676T	Q226-TAA stop	Leaky
	vanB9251	T381C	Silent	
ADP9253	*vanB9253*	T174A	C58-TGA stop	Null

[a] A genotypically identical strain was isolated after a separate round of mutagenesis.

not destroyed at the elevated temperature and attains a conformation that allows it to resume activity at the lower temperature.

Many of the amino acid substitutions in the mutant VanA and VanB proteins are in primary-structure segments homologous to regions known to have important functions in other oxygenases. In VanA, D61G presumably disrupts the structure of the Rieske iron-binding site (Fig. 4). Five amino acid substitutions are clustered in the iron-binding active site, although only one of these (H156R) substitutes an amino acid that ligates iron (Fig. 4). The significance of N150 and L151 is highlighted by three amino acid substitutions (N150H, N150D, and L151Q) that cause a null phenotype (Fig. 4). As described above, the amino acid substitution W217R causes a temperature-sensitive phenotype but does not lead to denaturation of the enzyme at the restrictive temperature.

The importance of the VanA primary structure between positions 199 and 228 is indicated by the clustering of five mutations in this protein segment (Fig. 4). Four of these mutations (W199R, C220R, C220W, and V228D) cause a null phenotype, and one mutation (W217R) leads to a temperature-sensitive phenotype. An additional mutation in this region, A224T, alters the phenotype of strains containing Q305R. By itself, the Q305R substitution in VanA produced strain ADP9203 (Table 1), a strain that grows slowly with vanillate. Its rate of growth is unaltered by exposure of cells to the vanillate analogs *m*-anisate, *m*-toluate, and 4-hydroxy-3,5-dimethylbenzoate. Growth of strain ADP9201 (which contains both A224T and Q305R in VanA; Fig. 3) is completely inhibited by the analogs. Single amino acid substitutions conferring this phenotype were Q306P in VanA and S35P in VanB (Fig. 5). Growth of the heat-sensitive strain ADP9204 containing the amino acid substitution W217R (Fig. 3 and 4) at the permissive temperature was inhibited by the vanillate analogs.

Four of the five observed single amino acid substitutions in VanB (Fig. 5) occur within peptide segments for which function can be inferred from the study of homologous enzymes. One substitution, I116F, replaces an amino acid in the presumed NAD ribose-binding site of VanB. The substituted isoleucine is conserved in many oxygenases, including toluene monosulfate monooxygenase and chlorobenzoate dioxygenase, yet the I116F substitution results in an enzyme that allows slow growth at both 22 and 37°C. Three of the five amino acid substitutions occur in the highly conserved iron-sulfur-binding site that extends over 12 residues in VanB (Fig. 5).

Nucleotide and amino acid substitutions in phenotypic revertants. Upon incubation in medium containing vanillate as the sole carbon source, strain ADP9200 gave rise to a second-

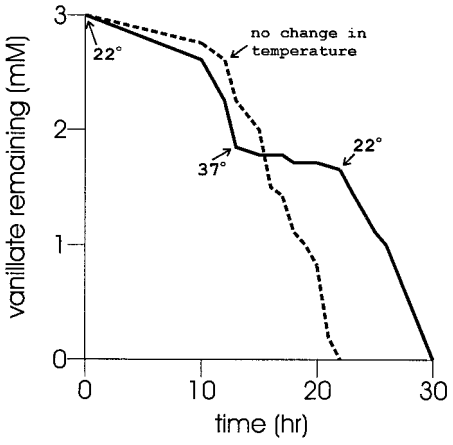

FIG. 3. Influence of temperature on vanillate removal by ADP9204 (*vanA9204*). Uninduced cells were incubated with vanillate at 22°C. At 13 h, the time indicated by the arrow labeled 37°C, one of the cultures was shifted to this temperature. The arrow labeled 22°C indicates that the culture was restored to this temperature at 22 h and metabolism of vanillate recommenced.

ary mutant strain, ADP9299, that grew rapidly with the compound. The Q306P amino acid substitution in VanA of strain ADP9200 replaced an amino acid that is conserved among vanillate demethylases, so it was anticipated that VanA strain ADP9299 would have undergone a P306Q reversion restoring the conserved amino acid residue. This was not the case. The mutation giving rise to strain ADP9299 causes a P306S substitution in VanA (Fig. 4; Table 2), indicating that there is some latitude in the amino acid substitutions that are tolerated in

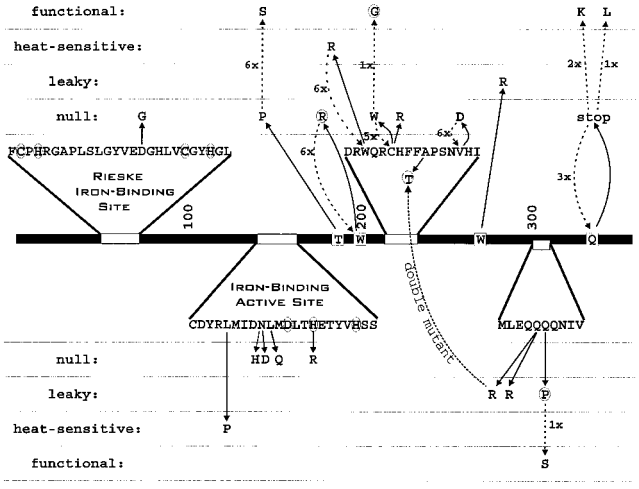

FIG. 4. PCR-induced mutations altering the activity of VanA. The primary structure of VanA is indicated as a dark bar with relevant portions of the amino acid sequence expanded. Shaded amino acid residues in the primary sequences indicate amino acids conserved in the Rieske iron-binding site and the iron-binding active site. Dark arrows indicate amino acid substitutions caused by PCR mutagenesis. As indicated by the vertical positioning of amino acid substitutions, most caused a null phenotype, as judged by the ability of mutant cells to grow with vanillate. Some of the sequenced mutations caused a heat-sensitive phenotype with respect to growth with vanillate, and some of the mutants were classified as leaky because they allowed slow growth with vanillate. Among the mutants with detectable enzyme function, three fail to grow with vanillate in the presence of vanillate analogs. Substituted amino acids causing this phenotype are surrounded by dotted circles. One of the three mutants had undergone a double mutation, and this is marked by a dotted line connecting the mutant amino acid residues. Mutations restoring *van* function are indicated by dashed arrows.

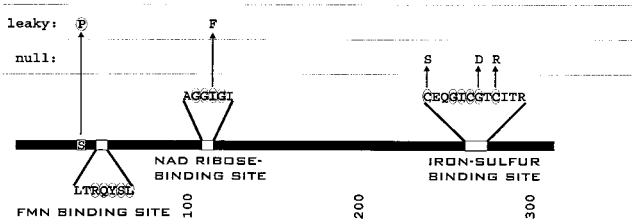

FIG. 5. PCR-induced mutations altering the activity of VanB. The primary structure of VanB is indicated as a dark bar with relevant portions of the amino acid sequence expanded. Shaded amino acid residues in the primary sequences indicate conserved amino acid sequences with the depicted functions. Dark arrows indicate amino acid substitutions caused by PCR mutagenesis. The dotted circle indicates an amino acid substitution that appears to increase the sensitivity of vanillate demethylase to competitive inhibition. FMN, flavin mononucleotide.

this position. This finding warranted further investigation of the degree to which amino acid substitution of conserved residues would yield a functional protein.

The initial target of this study was the stop codon substituting the wild-type codon for Q328 in VanA of strain ADP9222 (Fig. 4; Table 2). The amino acid residue was of interest because it is conserved among oxygenases as distant as *Comamonas testosteroni* toluene monosulfate oxygenases (17), which has an amino acid sequence identity of only 33% with the *Acinetobacter* enzyme. DNA containing *vanA* from strain ADP9222 was mutagenized by PCR amplification, and mutations that restored the ability to grow with vanillate were selected after strain ADP9222 had been transformed with this DNA. The nucleotide sequences of *vanA* from six of these phenotypic revertants were determined, and three of the strains had undergone true stop328Q reversion. However, as shown in Fig. 4 and Table 2, in one of the phenotypic reverants (stop328L), the original amide residue was replaced with a hydrophobic residue and two of the revertants (stop328K) contained a basic residue at this position. It thus appears that conservation of the glutamine in the widely divergent proteins is not the consequence of direct selective pressure for its function. Toleration of limited variation was demonstrated by consistent substitution of one alcohol side chain for another, as phenotypic reversion of T186P was achieved by P186S.

Three mutated residues in VanA were invariably restored to the wild type after six separate treatments with PCR-mutagenized DNA. The respective R199W, R217W, and D228V reversions indicate that the wild-type residues are under some selective constraints (Fig. 4; Table 2). True reversion of the C220W mutation was observed in five cases, but one phenotypic revertant contained a glycyl residue rather than the wild-type cysteinyl residue, which is conserved in all known vanillate demethylase sequences (Fig. 4; Table 2). Growth of strain ADP9307 containing the glycyl substitution was inhibited by vanillate analogs, suggesting that this mutation alters the affinity of the enzyme for its substrate.

DISCUSSION

The substrate range of the vanillate demethylase shows that this enzyme, like *p*-toluenesulfonate monooxygenase (23), catalyzes demethylation or monohydroxylation with a variety of different aromatic substrates. Catalysis appears to depend upon the presence of a carboxyl group and a methyl or methoxy substituent in the *meta* position, allowing a nucleophilic attack (Fig. 2); three analogs (*m*-anisate, *m*-toluate, and 4-hydroxy-3,5-dimethylbenzoate) inhibited the activity of the demethylase. The activity of the enzymes involved in the degra-

TABLE 2. Phenotypic revertants of *vanA* mutants

Parental strain	Phenotypic revertant	Gene designation	Nucleotide substitution	Amino acid substitution	No. of isolates	Phenotype
ADP9200		*vanA9200*	A917C	Q306P		
	ADP9299	*vanA9299*	C916T	P306S	1	Wild type
ADP9204		*vanA9204*	T649A	W217R		
	Wild type		A649T	R217W	6	Wild type
ADP9207		*vanA9207*	T660G	C220W		
	Wild type		G660T	W220C	5	Wild type
ADP9207		*vanA9207*	T660G	C220W		
	ADP9307	*vanA9307*	T658G	W220G	1	Increased affinity for inhibitors
ADP9210		*vanA9210*	T683A	V228D		
	Wild type		A683T	D228V	6	Wild type
ADP9210		*vanA9210*	T683A	V228D		
	ADP9310	*vanA9210*	A394T	S132C	1	Wild type
ADP9211		*vanA9211*	T595C	W199R		
	Wild type		C595T	R199W	6	Wild type
ADP9213		*vanA9213*	A556C	T186P		
	ADP9313	*vanA9313*	C556T	P186S	6	Wild type
ADP9222		*vanA9222*	C982T	Q328stop		
	Wild type		T982C	Stop328Q	3	Wild type
ADP9222		*vanA9222*	C982T	Q328stop		
	ADP9322	*vanA9322*	T982A	Stop328K	2	Wild type
ADP9222		*vanA9222*	C982T	Q328stop		
	ADP9323	*vanA9323*	A983T	Stop328L	1	Wild type

dation of protocatechuate was not affected by any of these compounds.

The distribution of PCR-generated mutations creating nonsense codons is, as expected, fairly random throughout *vanA* and *vanB* (Table 1). The null phenotype caused by termination at residue 328 demonstrates that the carboxy-terminal 31 amino acid residues of this protein are required for activity, and termination codons at earlier sites in the sequence presumably disrupt vanillate demethylase activity by giving rise to even smaller protein products. Of particular interest are three stop codons, two in *vanA* and one in *vanB*, that result in a leaky phenotype (Table 1). Evidently, the translational context (24, 27, 38) of these codons allows readthrough sufficient to permit slow utilization of vanillate.

The selected missense codons either replace essential amino acid residues or perturb the protein's structure so that it loses function. An example of the former is H156R, substituting an iron-binding ligand in VanA, and an example of the latter is the W217R substitution, creating a protein that is functional at 22°C but not at 37°C (Fig. 3). Clustering of missense mutations within specified segments of the primary structures of VanA and VanB is significant because it highlights contributions of these segments to enzyme function. The five missense mutations recovered in VanB occur either within or near portions of the primary structure that can be assigned functions on the basis of sequence comparison with related proteins (Fig. 5). Similarly, most of the recovered missense mutations are clustered in four primary-structure regions in VanA. Two of these have well-defined functions in the binding of iron, and the functions of the other two regions are less clear (Fig. 4). Some mutations in the latter two regions appear to increase the affinity of the enzyme for inhibitors (Tables 1 and 2; Fig. 4), suggesting that these regions contribute to substrate binding, a function known to be associated with VanA. The finding that cells containing S35P in VanB appeared to be sensitive to competitive inhibition by vanillate analogs raises the possibility that VanB makes a contribution, possibly indirect, to substrate binding.

The fact that relatively few of the selected missense mutations occur outside of clusters where function can be inferred suggests that vanillate demethylase can accumulate amino acid substitutions at many sites without losing function. A direct approach to understanding amino acid residues that can be tolerated at a specified position is the use of PCR mutagenesis to determine amino acid substitutions that allow phenotypic reversion of a known mutation (19). In the present study, flexibility was indicated by phenotypic reversion of a stop codon resulting in functional proteins with overall Q328K and Q328L substitutions (Table 2; Fig. 4). Therefore, the demonstrated conservation of glutamine at this position in widely divergent oxygenases cannot be taken as evidence of severe functional constraints. The importance of W199, W217, and V228 was underlined by the fact that activity was restored to mutants with substitutions of these residues only by direct reversion to the wild type (Table 2; Fig. 4). The same general pattern was observed with reversion of C220W, but in a single instance, a functional protein was formed by substituting a glycyl residue for the original cysteinyl residue at this position. Intriguingly, the protein containing this amino acid substitution was relatively sensitive to competitive inhibitors of vanillate demethylase, reinforcing the interpretation that this portion of the protein may contribute to substrate binding.

In one case, strain ADP9310 (Table 2), the consequence of a null mutation (V228D in VanA) overcame a mutation altering an amino acid (S132C in VanA) that is distant in the primary sequence. This finding raises the possibility that the different amino acid residues affected by mutation is within physical proximity in the folded protein. Observation of the second-site mutation was nearly masked by the predominance of six strains in which the V228 mutation had reverted to the wild type (Table 2). Therefore, additional evidence gained by probing such second-site mutations would be sharpened by seeking revertants created by PCR mutagenesis with primers excluding the primary mutation.

The most remarkable phenotypic revertants were those obtained after mutagenized DNA restored the wild-type phenotype to strains containing the T186P substitution in VanA (Fig. 4; Table 2). In each of six independent isolates, wild-type

activity had been restored not by true reversion but by a different substitution at the same nucleotide position causing a P186S amino acid substitution. It seems that the alcohol functions of threonine and serine side chains are both important and interchangeable in VanA; indeed, both residues are found at position 186 in sequenced vanillate demethylases. The remarkable observation is the preferential substitution by secondary mutation of serine for the original threonine, and the basis for this may lie in the nature of the nucleotide substitutions that were available for selection. The initial nucleotide substitution is a relatively unusual transversion, A556C, in strain ADP9213, whereas the nucleotide substitution giving rise to the phenotypic revertant is the relatively frequent transition C556T (Table 2). This finding illustrates that it would be unwise to regard nucleotide substitutions as entirely random when charting the course of amino acid substitutions in evolution.

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