Genetics of ferulic acid bioconversion to protocatechuic acid in plant-growth-promoting *Pseudomonas putida* WCS358

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Transposon Tn5 genomic mutants of plant-growth-promoting *Pseudomonas putida* strain WCS358 have been isolated which no longer utilize ferulic and coumaric acids as sole sources of carbon and energy. Genetic studies confirmed previous biochemical data showing that ferulic acid is degraded via vanillic acid, and coumaric acid via hydroxybenzoic acid. The genes involved in these enzymic steps were cloned and characterized. Two proteins designated Fca (26.5 kDa) and Vdh (50.3 kDa) were identified as responsible for the conversion of ferulic acid to vanillic acid; the proteins are encoded by the *fca* and *vdh* genes which are organized in an operon structure in the chromosome. The Vdh protein is 69% identical at the amino acid level to the Vdh protein recently identified in *Pseudomonas* sp. strain HR199 and converts vanillin to vanillic acid. Homology studies revealed that the Vdh proteins exhibited significant identity to aldehyde dehydrogenases from different organisms whereas Fca belonged to the enoyl-CoA hydratase family of proteins. Two proteins, designated VanA (39.9 kDa) and VanB (34.3 kDa), encoded by two genes, *vanA* and *vanB*, are organized in an operon in the chromosome. They were found to be responsible for the demethylation of vanillic acid to protocatechuic acid. The VanA proteins showed no homology to any other known protein, while VanB belonged to the ferredoxin family of proteins. This two-component enzyme system demethylated another phenolic monomer, veratric acid, thus indicating broad specificity. Studies of the regulation of the *vanAB* operon demonstrated that the genes were induced by the substrate, vanillic acid; however, the strongest induction was observed when cells were grown in the presence of the product of the reaction, protocatechuic acid.

Keywords: *Pseudomonas*, ferulic acid, coumaric acid, vanillic acid, regulation

INTRODUCTION

Pseudomonads have remarkable catabolic activity as they are able to utilize a wide variety of low-molecular-mass compounds as unique sources of carbon and energy (Sokatch, 1986 and references therein). For example, phenolic monomers such as *trans*-ferulic, *p*-coumaric and vanillic acids (Fig. 1) are readily degraded by many *pseudomonads*, which use them as the sole carbon source. These aromatic compounds arise abundantly in the environment from the biodegradation of

lignin, which is predominantly accomplished by white rot fungi (Kirk & Farrell, 1987). Interestingly, several aromatic compounds related to lignin (including ferulic acid, vanillin, vanillic acid, *p*-hydroxybenzoic acid and protocatechuic acid) are known to be found in intact and wounded plant cells and are abundantly present in the rhizosphere. These compounds, amongst others, are important for signalling in gene expression between plants and some bacteria, as, for example, in *Agrobacterium tumefaciens* (Bolton *et al.*, 1986; Melchers *et al.*, 1989), highlighting a possible role of these compounds in the communication between plants and beneficial rhizosphere bacteria.

The EMBL/GenBank/DBJ accession numbers for the sequences reported in this paper are Y14772 (*fca/vdh*) and Y14759 (*vanA/vanB*).

In bacteria, diverse aromatic compounds are most commonly degraded in the initial conversion steps to a limited number of central intermediates, namely catechol (or substituted derivatives) and protocatechuic acid. These intermediates are then channelled into two possible ring fission pathways, either the *ortho*- or *meta*-cleavage pathway, funnelling these compounds into the tricarboxylic acid cycle (van der Meer *et al.*, 1992). The genes and enzymes and the gene regulation of these two pathways have been extensively studied in various Gram-negative soil bacteria (Parke, 1995 and references therein). The biochemical mechanism of ferulic acid degradation by bacteria was first reported by Toms & Wood (1970), where they established that vanillin, vanillic acid and protocatechuic acid are intermediates before ring fission in *Pseudomonas acidovorans*. Similarly, Delneri *et al.* (1995) showed that ferulic and coumaric acids were degraded to protocatechuic acid via vanillic acid and hydroxybenzoic acid, respectively, in *Acinetobacter calcoaceticus*. These initial conversion steps of ferulic acid involve the shortening of the side chain by a two-carbon fragment to yield vanillic acid followed by demethylation producing protocatechuic acid (Fig. 1), which undergoes ring fission by the *ortho* pathway. The genetics of the pathway from vanillic acid to protocatechuic acid have been described by Brunel & Davison (1988) and also recently by Priefert *et al.* (1997). Both studies showed that two proteins (VanA and VanB) form a vanillate demethylase complex which demethylates vanillic acid to protocatechuic acid. VanA has no significant homology to any known protein and is believed to be a monooxygenase, whereas VanB appears to be related to ferredoxins. Priefert *et al.* (1997) have also identified an aldehyde dehydrogenase gene which is responsible for the conversion of vanillin to vanillic acid.

In this study, we present the genetic determinants for the degradation of ferulic acid to protocatechuic acid in plant-growth-promoting *Pseudomonas putida* strain WCS358. A Tn5 genomic mutant bank was used to investigate the operonic structure of genes involved in this degradation. The regulation of expression of one operon is reported and the possible role of all these identified genes in rhizosphere colonization is discussed.

METHODS

Strains, plasmids and media. Strains used in this study include *Escherichia coli* DH5 α (Hanahan, 1983), XL1-Blue (Bullock *et al.*, 1987) and JM101 (Messing, 1983). *Pseudomonas putida* WCS358 is a plant-growth-promoting strain isolated from the rhizosphere of potato roots (Geels & Schippers, 1983).

E. coli was grown in LB medium (Miller, 1972) at 37 °C, whereas *P. putida* WCS358 was cultured in LB medium at 30 °C or in M9 minimal medium (Maniatis *et al.*, 1982). When ferulic acid, vanillic acid, veratric acid and protocatechuic acid were used as carbon source they were added to the growth medium at a final concentration of 0.15%. The following antibiotic concentrations were used: tetracycline, 10 $\mu\text{g ml}^{-1}$ (*E. coli*) and 40 $\mu\text{g ml}^{-1}$ (strain WCS358);

kanamycin, 50 $\mu\text{g ml}^{-1}$; nalidixic acid, 25 $\mu\text{g ml}^{-1}$; chloramphenicol, 25 $\mu\text{g ml}^{-1}$ (*E. coli*) and 250 $\mu\text{g ml}^{-1}$ (strain WCS358). Ampicillin (50 $\mu\text{g ml}^{-1}$) and spectinomycin (25 $\mu\text{g ml}^{-1}$) were used for *E. coli*.

Plasmids used in this study are listed in Table 1. Plasmid pICFERTn5 is an *EcoRI* fragment containing transposon Tn5 and bordering DNA from the chromosome of genomic mutant FA11 cloned in the *EcoRI* site of plasmid pIC20R. The same *EcoRI* fragment cloned in the *EcoRI* site of pMP220 yielded pMPFERTn5.

Analysis of substrate metabolism. The biodegradation of ferulic acid by *P. putida* WCS358 and the Tn5 mutants was analysed by reverse-phase HPLC (RP-HPLC), using a Varian 9010 solvent delivery system equipped with a Varian 9050 UV/vis detector. Mutants were grown at 30 °C in LB medium supplemented with 0.15% (w/v) ferulic acid. Samples were withdrawn from cultures after 12 and 36 h growth and centrifuged at 12000 g. The supernatant was diluted 100-fold into methanol and filtered through 0.2 μm filters; 10 μl samples were loaded on a 5 μm spherical C18 reverse-phase column (Supelcosil LC18 150 $\times$ 4.6 mm; Supelco) and eluted with 35% methanol and 65% water with 0.1% acetic acid, at a flow rate of 0.8 ml min $^{-1}$. The eluted metabolites were detected at 279 nm. The culture by-products were identified by comparing their elution time with those of pure standards. Acetic acid was detected and quantified with an enzyme analysis kit from Boehringer Mannheim.

Recombinant DNA techniques. Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end-filling with Klenow fragment of DNA polymerase, hybridizations, radioactive labelling by random priming and transformation of *E. coli* were performed as described by Maniatis *et al.* (1982). Southern hybridizations were performed using Hybond-N+ (Amersham). Plasmids were purified as described by Birnboim (1983), and with Qiagen columns. Total DNA from *Pseudomonas* strains was isolated by Sarkosyl/Pronase lysis as described by Better *et al.* (1983). Triparental matings between *E. coli* and *Pseudomonas* were performed with the helper strain *E. coli*/pRK2013 (Figurski & Helinski, 1979).

Reporter gene fusion assays. β -Galactosidase activity was determined essentially as described by Miller (1972) with the modifications of Stachel *et al.* (1985).

Isolation of mutants unable to use ferulic and vanillic acids as carbon source and their complementation with a *P. putida* WCS358 gene bank. Mutants unable to use ferulic acid and vanillic acids as carbon sources, but retaining the ability to use protocatechuic acid, were selected in the following way. Purified colonies from a Tn5 genomic mutant bank constructed using *E. coli* S17-1/pSUP2021 (Marugg *et al.*, 1985) were grown at 30 °C in replicate on minimal M9 plates containing 50 μg kanamycin ml $^{-1}$ and either ferulic, vanillic or protocatechuic acid. Mutants were selected which were unable to grow on ferulic and vanillic acids but able to grow on protocatechuic acid, and were complemented for their defective growth as follows. A genomic library of *P. putida* WCS358 made in *E. coli* JM101 from a partial *HindIII* digest ligated into cosmid pLAFR3 was used. Triparental matings between this library, an *E. coli*/pRK2013 mobilizer (about 4×10^9 cells of each) and each strain WCS358 Tn5 mutant (2×10^8 cells) were set up on 0.45 μm membrane filters (Millipore) on LB plates. After 12 h incubation at 30 °C, cells were resuspended and plated on minimal M9 plates containing the appropriate carbon source with tetracycline (40 $\mu\text{g ml}^{-1}$)

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pRK2013	Km ^r Tra ⁺ Mob ⁺ , ColE1 replicon	Figurski & Helinski (1979)
pSUP2021	<i>tnpA</i> ⁺ , pACYC184 replicon, Cm ^r	Stachel <i>et al.</i> (1985)
pIC20R	ColE1, Ap ^r	Lawrence-Marsh <i>et al.</i> (1984)
pBluescript II SK	ColE1, Ap ^r	Stratagene
pBluescript II KS	ColE1, Ap ^r	Stratagene
pPH1JI	IncP1, Gm ^r	Beringer <i>et al.</i> (1978)
pMP220	Promoter probe vector, IncP, Tc ^r	Spaink <i>et al.</i> (1987)
pLAFR3	Broad-host-range cloning vector, pRK290 derivative, IncP1, Tc ^r	Staskawicz <i>et al.</i> (1987)
pCOSE1	pLAFR3 containing <i>P. putida</i> DNA	This study
pCOSI1	pLAFR3 containing <i>P. putida</i> DNA	This study
pCOSVA4	pLAFR3 containing <i>van</i> genes	This study
pBVA4	pBluescript II SK containing <i>van</i> genes	This study
pVABPR	pMP220 containing the promoter of the <i>vanAB</i> operon	This study
pSKI1	pBluescript II KS containing <i>fca/vdh</i> genes	This study
pICFERTn5	pIC20R containing <i>fca/vdh</i> genes with a Tn5 insertion	This study
pMPFERTn5	pMP220 containing <i>fca/vdh</i> genes with a Tn5 insertion	This study

and kanamycin (50 µg ml⁻¹). Plates were incubated for 2 d at 30 °C and transconjugants which regained the ability to grow on the appropriate carbon source were scored.

Site-specific exchange of mutations with the *Pseudomonas* chromosome. Target plasmid pMPFERTn5, carrying the *fca/vdh* genes with a Tn5 insertion, was homogenized with the corresponding region of the genome of *P. putida* WCS358 by a marker-exchange procedure described by Corbin *et al.* (1982) and Venturi *et al.* (1995). pPH1JI was used as the incoming IncP1 incompatible plasmid, and selections were made on LB plates containing nalidixic acid (25 µg ml⁻¹), gentamicin (40 µg ml⁻¹) and kanamycin (50 µg ml⁻¹). Putative marker-exchanged mutants were streaked on LB plates containing tetracycline (40 µg ml⁻¹) to confirm loss of IncP1 recombinant plasmids.

DNA sequence determination and analysis. DNA fragments from plasmid pBVA4 harbouring the *van* genes were prepared by digestion with *Bam*HI, *Cl*aI, *Xho*I and *Sma*I and cloned directly in pBluescript II SK and pBluescript II KS. DNA fragments from plasmid pSKI1 harbouring the *fca* and *vdh* genes were prepared by digestion with *Nru*I, *Stu*I, *Eco*RI, *Bgl*II and *Cl*aI and cloned directly in pBluescript II SK and pBluescript II KS. The constructs were either encapsidated as single-stranded DNA upon infection with helper phage VCSM13 (Stratagene) or used directly for DNA sequencing. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using [³⁵S]dATPαS for labelling and 7-deaza-dGTP (Pharmacia) instead of dGTP. The nucleotide sequence was determined in both directions and across all restriction sites.

RESULTS

Isolation and characterization of *P. putida* WCS358 mutants

P. putida strain WCS358 can utilize ferulic, coumaric and vanillic acids as the sole carbon source. The first steps in the degradation of ferulic acid in *P. putida* most probably involve the conversion to vanillic acid followed

by the demethylation to protocatechuic acid (see above and Fig. 1). To test this, we isolated *P. putida* Tn5 mutants unable to carry out these conversion reactions. Ten thousand genomic Tn5 mutants were screened for their ability to grow on either ferulic, vanillic or protocatechuic acid, and 15 mutants, designated FAI1–FAI15, which could not grow on ferulic acid but grew on protocatechuic acid as sole carbon source were isolated. One of the mutants (FAI15) was also unable to grow on vanillic acid. Interestingly, mutants FAI1–FAI14 were not able to degrade *p*-coumaric acid, which was consistent with the pathway shown in Fig. 1. All the other 14 mutants grew on vanillic acid as sole carbon source.

In this study, we concentrated on the further characterization of mutants FAI1 and FAI15. HPLC analysis of supernatant samples from *P. putida* WCS358 and the 15 Tn5 genomic mutants showed that the wild-type completely catabolized ferulic acid whereas mutants FAI1–FAI14 did not convert ferulic acid to any other aromatic compound. Tn5 mutant FAI15 transformed ferulic acid to vanillic acid, as expected, and was deficient in the demethylation of vanillic acid to protocatechuic acid (results not shown).

Cloning of genes involved in the first step(s) of ferulic acid degradation

A genomic library of strain WCS358, cloned in *E. coli* JM101 using cosmid vector pLAFR3, was introduced into mutant FAI1. Transconjugants showing complementation of this mutation were selected for by growth on M9 plates containing ferulic acid. Several cosmids were purified from such transconjugants and were used separately to transform *E. coli* DH5α. Subsequently, these plasmids were transferred by conjugation into mutant FAI1, confirming that they complemented the mutation for growth on ferulic acid.

Fig. 1. Biodegradation pathway of *trans*-ferulic and *p*-coumaric acids. Proposed mutations in the chromosome of the identified genomic mutants and the genes involved in each step which have been identified and characterized in this study are indicated. Genomic Tn5 mutants FAI1–14 were blocked as indicated and FAI15 was unable to demethylate vanillic acid (see text for details).

Fig. 2. (a) Physical map of the 25 kb *Hind*III fragment in pCOSI1 and of the 4.9 kb *Xho*I–*Hind*III region which contains the *fca/vdh* operon. The arrow represents the direction of transcription of the two genes. (b) Physical map of the 4.1 kb fragment of *P. putida* WCS358 chromosomal DNA in pCOSVA4 containing the demethylating genes *vanAB* as indicated. The arrow represents their direction of transcription.

Restriction analysis revealed that all cosmids contained a single *Hind*III fragment of approximately 25 kb, and the complementing cosmid was designated pCOSI1. Cosmid pCOSI1 also complemented the other 13 Tn5

mutants (FAI2–FAI14), restoring their ability to grow on ferulic acid as sole carbon source.

To map the ferulic acid catabolic gene(s) within this 25 kb fragment, Tn5 and its flanking DNA were cloned from mutant FAI1. Chromosomal DNA purified from mutant FAI1 was completely digested with *Eco*RI (does not cut Tn5) and fragments larger than 5 kb were purified by agarose gel electrophoresis and ligated into the *Eco*RI site of plasmid pIC20R. After transformation of *E. coli* DH5 α , transformants which were ampicillin and kanamycin resistant (Tn5 carries the kanamycin-resistance gene) were identified. This resulted in the isolation of a plasmid designated pICFERTn5, which contained a 9 kb *Eco*RI fragment including Tn5. Southern hybridization and restriction analysis (data not shown) between pCOSI1 and pICFERTn5 revealed that they contained homologous DNA fragments, presumably the same locus. Fig. 2(a) shows the location of Tn5 as mapped in pCOSI1.

To test further whether the 25 kb fragment in pCOSI1 was responsible for the observed phenotype, we re-cloned the *Eco*RI fragment from plasmid pICFERTn5 in the broad-host-range plasmid pMP220 (yielding pMPFERTn5) and transferred it to *P. putida* strain WCS358. This latter plasmid was used in a marker-exchange experiment (Corbin *et al.*, 1982) to introduce site-specific mutations within this locus in the WCS358 genome. The fidelity of the marker exchange was confirmed by Southern hybridization analysis (results not shown). This experiment resulted in the construction of mutant FAI1' which was unable to grow on ferulic acid as sole carbon source. It was concluded that all mutants had a Tn5 integration in the same region of the chromosome and that this locus was involved in ferulic acid catabolism in *P. putida* WCS358.


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1  ACCTCGGCGAGCGCCAGCGCGGGGATGGTAACCCGCTGCTGCTGGCGTCGACAACTGCTCGGTAAAGCTCAACAGCTCGGCGAGGCGCAGACGGCA
2  TGGCAGGTACCCCGAGCGAGCGGATCATGCGCGCGCGCAAGCGCTGTGCTGAAAGCAAGGTCGCGCCCAATCAGCTGCTGATCAGGTGTACT
101 GCTGAACACACGAGCGGATGCTTCGCGTCGCCAGCGCGCGCAGGACGATTCCGAGCGCTATCGAGCGGTTCACCGTCAATCAGGATGCGGCCACTGTC
201 GGCACGGTCCAGGCGCTGGCAATCAGGTGAGCAACGCTGCTTGGCAGTGGCTGCGACTCCCCATCAGCGCGAGGCTGCTGCGCGCGCGCAGGCGACAGG
401 TCGATGCCCTGACAGACCGGAGCGGCGCTTGGGCGGTGATGAACGACTTGTGCGAGTGTGCGACCGACGATCGGGAGTGTTCCTGCTGGCGCCACCT
501 CGCAAGCGTGGCTGAGTGGCCCTTAGGAATAGCACAGCCAGGCACTCAACAGCCAGGCAAGGAGCTAATGGGATCCATTAAATAAAACAAATGGCT
601 ACTCCGAGAGCGCTCTGTGATGAGCAAGCACTTGTGATGTGCGCTGACCGCTGACCGAATTCGCCGACAGCGCTTGGCGCGCAATCTCGCGGAAA
       N Y P R N T W Y V A C T P D E I A D K P L G R Q I C G E K
701 AGATCGTGTCTACAGAGGCGCACGAGACGGGTGGCGCGCTGGAAGATTTTTGGCGCACCGCGCGGCGCACTGCTGCTGGGCTATGTCGAAACGGCAA
       I V F Y R G H E N R V A A V E D F C R T R A P L S L G Y V E N G N
801 CCGTGTGTGTGCTGACCTGCTTGTGTAATGCGCTGCGCGCGCAAGACTGTGAGATGCCAGCGCAACGGGTGAGGCGCTTCCCTGCAACAGACTTTC
       L V C G G Y E G L V M G C D G K T V E H P G Q R V R G F P C N K T F
901 GCGTGTGTGAGCGCTATGGCTTCATCTGGGTGTGGCGCGCGCACAGGCAAGGCAAGCGCTGATCCCGCATCTGGAATGGCGCTAAACGATG
       A V V E R Y G G F I W V M P G D Q A Q A D P S L I P H L E W A V M D D
1001 ACTGGCGCTATGAGCGCGCGGTGTGTCACATCGCTGCGCTGAGTATCGACAACTGATGAGCTGACCGCTGACCGCTGACCGCTGACCGCTGACCGCT
       N A Y G G G L F H I G C D Y R L N I D F L N D L T R E T Y V H A S
1101 CAGCATCGCGCAGAAAGAAATCGATGAAGACCGCGCTACCAACCGTACCGCGCGAGAGTCTGAGTGCACGGCAGATGGAAACATCATGGCAGCGCG
       S I G Q K E I D E A P P Y H R H R R E V L S A R H M E N I M A P P
1201 TTCTGGCGCTATGCGCTTGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       F W R N A L R G H G L A D D V P V E R W Q I C R F T P P S E V L I E
1301 AAGTCGCGTGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       V G V A E A T G G K G G Y H A E Q H K A S S I V V W N F I T P E T D T
1401 CTCGATCTGATCTCTCTGAGTATTGGCGCTGCGCTGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       S I W Y F W V L A C N F A A Q D Q A L T A N I R E Q Q G K I F S E
1501 GACCTGGAATGCTCGAAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       D L E R L E R L L A Y P Q R N L L K L H I D A G G Y Q S R K
1601 AGATCTCTGAGCGCGCTGATCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       I L E R L I A N E R M P Q A E L I A T S A S P A M I D A
1701 CGTAGTGTGTTCCCGTAACGAGCGAGCACAGCATCTGCGCTTGAAGTGGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       V V V S R N D E A Q D I C S F E L A A V D G S L L R P S A G A R I
1801 GATGTGCACTGCTGCGTGAAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       D V H L P E G Q V R Q Y S L C N H P E E R H R Y L I G V L K D P A S
1901 CAGCGCGCGGTTCGCGCGCGTTCGACGAGCGAGTCCACAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       R G G S R S L E R I H N G A R L R I S A P R H L F P L A Q G A R
2001 CCGCAGCGCTGTTGTCGAGCGCGTATCGCGATCACCGCGCGCTGTGCTGATGCGTGAAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       R S L L F A G G I G I T T P I L C N A E Q L A A S A D F E L H Y C A
2101 CGCTCCAGCGAAGCGCGCGCTTCATCGAGCGTATGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       R S S E R A A P I E R M R G A A F A D R L F V H F D E Q P E T A L D
2201 ACATCGCCCGAGGTGTTGGCGAAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       I A Q V L A N P Q A D V E L Y V C G P G G F M Q E V L E S A K A Q
2301 GGGCTGGCAGGAGCGTGTGCTGACGCGGAAATCTTTGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       G W Q E A C L E R I F A A P V D T Q G D G S F S V Q L N S T C G
2401 CAGGTGTTGAGGTGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       Q V F E V P A D Q S V V H V L E Q H G I A I A M S C E Q O I C G T C
2501 GCTTACAGCGCTGCTGCTGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG
       L T R V L S G T P E A S R P V F L T E Q E Q A L H D Q F T P C C S
2601 CGGTCGAAACCGCGCGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
       R S K T P L L V L D L
2701 TCTCGGCTAAGCGAAGTGGCTTGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
2801 GCATGTGGCGCTAGTGAAGCGCGCTATCCAGCGCGCTATGCGCGTACATCGAGCGCGAT 2864

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Fig. 4. Nucleotide sequence of the *vanA* and *vanB* genes. Nucleotides are numbered from the 5'-end; the predicted amino acid sequence for the two genes is given in single-letter code below the DNA sequence. The restriction sites used for sequencing, the potential Shine–Dalgarno sequences (underlined), and the position and direction of the three primers (1, 2 and 3) used for PCR amplification (see text for details) are shown. Double-underlined amino acids indicate the putative iron–sulphur binding region found at positions 267–271 of the VanB protein. Amino acids which appear in bold are identically conserved between the three VanAB proteins from *P. putida* WCS358, *Pseudomonas* sp. HR199 and *Pseudomonas* sp. ATCC 19151.

acid as sole carbon source. Therefore, it was concluded that the vanillic acid demethylating gene(s) was located within this fragment (Fig. 2b).

Nucleotide sequence of the vanillic acid (*van*) catabolic genes

The nucleotide sequence of the 4.1 kb *Hind*III fragment in plasmid pCOSVA4 was determined, and analysis revealed two ORFs as shown in Figs 2(b) and 4. The two ORFs appeared to be organized as an operon with a putative promoter upstream (see below) and both ORFs were preceded by a potential translational start sequence (Fig. 4). No potential transcriptional termination sequence was detected. To verify that genomic mutant FAI15 contained a transposon Tn5 insertion in this region, PCR was performed on purified FAI15 genomic DNA using primers 1 and 3 (Fig. 4), and resulted in amplification of a 7.5 kb DNA fragment. PCR with the genomic DNA of strain WCS358 as template and the same primers produced a 2.5 kb amplification product. It was concluded that the additional 5 kb in mutant FAI15 was due to Tn5.

Sequence analysis of the two ORFs revealed significant similarity with VanA and VanB (Fig. 4) of *Pseudomonas* sp. ATCC 19151 (Brunel & Davison, 1988) and with the VanA and VanB proteins of *Pseudomonas* sp. HR199 (Priefert *et al.*, 1997). The identity of VanA from strain WCS358 was 72 % and 75 % with the VanA protein of strain ATCC 19151 and HR199, respectively, while that of VanB from strain WCS358 was 57 % and 60 % with VanB of strain ATCC 19151 and HR199, respectively. Fig. 4 highlights the amino acids that are conserved in all three VanA and VanB proteins identified. This high degree of homology is not surprising as the VanAB proteins are also responsible for the demethylation of vanillic acid to protocatechuate in *Pseudomonas* sp. ATCC 19151 and *Pseudomonas* sp. HR199. The other *vanAB* genes are also organized in an operon where *vanA* is the first gene.

Regulation of the *vanAB* operon

Regulation of the *vanAB* operon was studied by cloning the putative promoter upstream of a promoterless β -galactosidase gene. Using two oligonucleotides, primers

Table 2. *P. putida* WCS358 *vanAB* promoter activity (β -galactosidase, Miller units) in *P. putida*, genomic mutant FAI1 and genomic mutant FAI15 in LB containing different aromatic compounds

Cells harbouring the plasmids were grown for 16 h. The medium was either LB or LB with the indicated aromatic acid added to a final concentration of 0.15% (w/v). All media were supplemented with 40 μ g tetracycline ml⁻¹. All measurements were done in triplicate; the mean value is given.

Strain*	β -Galactosidase activity on:			
	LB	LB + ferulic acid	LB + vanillic acid	LB + protocatechuic acid
WCS358(pMP220)	81	76	86	75
WCS358(pVABPR)	73	70	250	710
FAI1(pVABPR)	70	77	200	730
FAI15(pVABPR)	81	97	240	690

* Plasmid pVABPR contains the *vanAB* promoter cloned in reporter probe vector pMP220 (see results section).

1 and 2 (Fig. 4), the promoter (the region 500 bp upstream of the start codon of the first ORF encoding VanA; see Fig. 4) was PCR amplified and cloned in the *Xba*I and *Nsi*I restriction sites of plasmid pMP220, yielding pVABPR. This was achieved by having extensions in the oligonucleotides which allowed the generation of these restriction sites in the PCR-amplified DNA fragment allowing the direct cloning in the corresponding sites in pMP220. Consequently, plasmid pVABPR consisted of the *vanAB* promoter cloned in the correct orientation upstream of a promoterless β -galactosidase gene.

The promoter probe construct was transferred to *P. putida* WCS358 and genomic mutants FAI1 and FAI15, and the resulting transconjugants, grown in LB media supplemented with 0.15% aromatic compounds, were assayed for β -galactosidase activity (Table 2). The promoter was most active when *P. putida* and the two mutants harbouring pVABPR were grown in the presence of protocatechuic acid. When grown in LB with or without ferulic acid, relatively low β -galactosidase activities indicated little or no promoter activity. However, when the three strains containing pVABPR were grown in the presence of vanillic acid, intermediate promoter activity was observed.

DISCUSSION

This is the first report, to our knowledge, where genetic and molecular data on the bioconversion of ferulic acid to protocatechuic acid and of coumaric acid to *p*-hydroxybenzoic acid are presented. Screening a Tn5 genomic mutant bank of plant-growth-promoting *P. putida* strain WCS358 resulted in the isolation of several mutants which could no longer utilize ferulic acid as sole source of carbon while they could still utilize protocatechuic acid. Genetic and biochemical characterization of the mutants revealed that this bio-

conversion involves two different genetic loci. One of these is responsible for the conversion of ferulic acid to vanillic acid (Tn5 genomic mutants FAI1–FAI14; see Fig. 1); this locus is an operon encoding two proteins designated Fca (26.5 kDa) and Vdh (50.3 kDa). The other locus is responsible for the demethylation reaction from vanillic acid to protocatechuic acid (FAI15; see Fig. 1); similarly it consists of an operon encoding two proteins called VanA (39.9 kDa) and VanB (34.3 kDa). The mutants which are blocked in the conversion of ferulic acid to vanillic acid are also unable to convert *p*-coumaric acid to hydroxybenzoic acid, demonstrating that the same gene(s)/enzyme(s) are involved in the transformation of two related compounds (Fig. 1).

Our data suggest that Fca and Vdh are the only proteins involved in the transformation of ferulic acid to vanillic acid since screening of the Tn5 mutant bank resulted in the isolation of mutants affected only in the *fca/vdh* operon. The Tn5 integration site of mutant FAI1 showed that it integrated in the *fca* gene, the first gene of the operon (Fig. 3). Using primers 1 and 2 as depicted in Fig. 3 via PCR we also demonstrated that all mutants had Tn5 insertions in the *fca/vdh* operon (data not shown). Toms & Wood (1970) reported that in *P. acidovorans* the reaction sequence involved in hydration of the *trans* double bond gives phenylpropionic acid as a transient intermediate, followed by aldolase cleavage to vanillin and acetate. Since the Fca-derived amino acid sequence contained an enoyl-CoA hydratase signature, *fca* could be involved in the hydration of ferulic acid forming the enolate, and the aldolase reaction could then either occur spontaneously or require an enzyme. We were unable to detect the release of acetic acid from the *vanAB* mutant FAI15 when fed ferulic acid. If released, it could be metabolized very quickly, making its detection difficult. From our data it is clear that the conversion of ferulic acid to vanillic acid liberates a compound which can be used as carbon source, since the

vanAB mutant FAI15 converted all the ferulic acid to vanillic acid and was able to grow, albeit poorly. The Vdh protein has only been very recently reported by Priefert *et al.* (1997) also from a *Pseudomonas* strain. Our data show that it is highly identical to the Vdh protein from *Pseudomonas* sp. HR199, being an NAD-dependent aldehyde dehydrogenase activity for vanillin. Priefert *et al.* (1997) expressed *vdh* in *E. coli* and confirmed that it catalyses the conversion of vanillin to vanillic acid. These observations are consistent with the possibility that Fca converts ferulic acid to vanillin and Vdh converts vanillin to vanillic acid.

The reaction catalysed by the VanA and VanB proteins involves monooxygenation to demethylate vanillic acid with NAD(P)H as electron donor, yielding protocatechuic acid and formaldehyde (Bernhardt *et al.*, 1975; Buswell & Ribbons, 1988). VanA from the three *Pseudomonas* strains shows no significant homology with any other proteins currently present in the protein databases. However, VanB displays significant homology with ferredoxin-type reductases containing an iron-sulphur centre (Mason & Cammack, 1992) and belongs to the [2Fe-2S] subgroup, even though it is an exception as it consists of 315 residues. An iron-sulphur binding motif was found near the C-terminus at positions 263–271 (Fig. 4), where binding is mediated by the three cysteines; the fourth cysteine in VanB involved in binding is likely to be at position 302 (Niedle *et al.*, 1991). VanB showed homology (36% identity) with the PDR protein of *Burkholderia cepacia*, a phthalate dioxygenase reductase (Correl *et al.*, 1992). It seems most likely that VanA is the monooxygenase and VanB is the NADH-dependent reductase of the enzyme system.

We investigated the biodegradability by the VanAB proteins of another phenolic compound, namely veratric acid. This compound is mineralized via protocatechuic acid, initially requiring two demethylation reactions as compared to vanillic acid which only requires one (Fig. 1). Interestingly, our attempts to isolate a Tn5 genomic mutant in the vanillic acid to veratric acid transformation failed, and as a result of this and the inability of mutant FAI15 to utilize veratric acid as sole carbon source (parent strain WCS358 grew on this substrate) we postulate that the VanAB proteins catalyse demethylation of both methoxy groups in this substrate.

The *vanAB* operon is expressed only when certain aromatic acids are present. We have cloned the promoter region upstream of a promoterless β -galactosidase gene and showed that when *P. putida* WCS358 and genomic mutants FAI1 and FAI15 harbour this construct the promoter was activated in the presence of vanillic and protocatechuic acids. When grown in the absence of these compounds there was no transcription from this promoter (Table 2). The highest level of β -galactosidase activity was detected when the strain was grown with protocatechuic acid, displaying a sevenfold higher activity compared to when it was grown in the presence of vanillic acid. It therefore appears that the latter substrate partially activates the promoter while the product of the

reaction, protocatechuic acid, strongly induces transcription of the genes. Interestingly, ferulic acid does not induce *vanAB* even though vanillic and protocatechuic acids are intermediates in the biodegradation. The reason for this is currently not known. These promoter assays in different growth media were also performed in the two mutants FAI1 and FAI15 which are blocked in the degradation at different places. In mutant FAI1 there was no transformation of ferulic acid (see above) and promoter expression in this background gave similar results to the wild-type, confirming that the inducers are most likely vanillic acid and protocatechuic acid.

All the phenolic compounds mentioned in this study are known to be constituents of intact and wounded plant cells as they are employed in lignin biosynthesis. Furthermore, ferulic, vanillic and protocatechuic acids, vanillin and hydroxybenzoic acid induce the transcriptional locus of the *vir* loci in *Ag. tumefaciens* (Bolton *et al.*, 1986; Melchers *et al.*, 1989). As *P. putida* strain WCS358 lives in the rhizosphere where these compounds are abundant, these compounds might be expected to have a role in signalling gene expression of plant-growth-promoting factors and in rhizosphere colonization.

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