Cloning and sequencing show that 4-hydroxybenzoate hydroxylase (PobA) is required for uptake of 4-hydroxybenzoate in *Rhizobium leguminosarum*

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Mutants of *Rhizobium leguminosarum* bv. *viciae* MNF300 and *R. leguminosarum* bv. *trifolii* WU95 unable to accumulate 4-hydroxybenzoate lack 4-hydroxybenzoate hydroxylase. The capacity of these mutants to take up and grow on 4-hydroxybenzoate was restored by a 2.0 kb *EcoRI*–*PstI* DNA fragment. This contained only one ORF which had over 60% DNA sequence similarity with the structural gene for 4-hydroxybenzoate hydroxylase (*pobA*) from *Pseudomonas* spp. and *Acinetobacter*. Reported effects of metabolic inhibitors and substrate analogues on the apparent uptake of 4-hydroxybenzoate have now been shown to be due to their direct effect on 4-hydroxybenzoate hydroxylase. We propose that uptake of 4-hydroxybenzoate is via a metabolic 'drag' mechanism dependent on the activity of the *pobA* gene product.

Keywords: *Rhizobium leguminosarum*, aromatic substrate uptake, 4-hydroxybenzoate, sequence of *pobA*

INTRODUCTION

Many bacteria degrade aromatic substrates via the catechol and protocatechuate branches of the 3-oxoadipate pathway (Stanier & Ornston, 1973). *Rhizobium leguminosarum* bv. *trifolii* (hereafter referred to as *R. trifolii*) TA1 possesses both pathways (Chen *et al.*, 1984) whereas *R. trifolii* WU95 and *R. leguminosarum* bv. *viciae* (hereafter referred to as *R. leguminosarum*) MNF300 have only the protocatechuate branch (Chen *et al.*, 1984). Growth of *R. leguminosarum* and *R. trifolii* on 4-hydroxybenzoate or protocatechuate requires enzyme induction; Chen *et al.* (1984) showed that 4-hydroxybenzoate hydroxylase and protocatechuate 3,4-dioxygenase were induced in the presence of 4-hydroxybenzoate. Growth on protocatechuate resulted in induction of protocatechuate dioxygenase, but not 4-hydroxybenzoate hydroxylase (Chen *et al.*, 1984; Wong *et al.*, 1991).

Despite detailed work on the catabolic enzymes involved in the 3-oxoadipate pathway, mostly in *Pseudomonas* (Stanier & Ornston, 1973; Wheelis, 1975; Yeh & Ornston, 1981; Shanley *et al.*, 1986; Hughes *et al.*, 1988; Entsch *et al.*, 1988; Van Berkel *et al.*, 1992) and

Acinetobacter (Neidle & Ornston, 1986; Doten *et al.*, 1987; Hartnett *et al.*, 1990; Neidle *et al.*, 1991; DiMarco *et al.*, 1993a, b), little is known about the systems involved in the entry into the cell of aromatic intermediates of this pathway. Although the presence of an uptake permease for 4-hydroxybenzoate (and other aromatic substrates) has been assumed for *Rhizobium* (Chen *et al.*, 1984; Wong *et al.*, 1991) as well as for other bacteria such as *Pseudomonas* spp. (Entsch *et al.*, 1988), there has been no unequivocal demonstration of such a permease. A benzoate permease has been reported in *Pseudomonas putida* (Thayer & Wheelis, 1982), but the question of whether 4-hydroxybenzoate enters rhizobia by diffusion (as originally assumed for *Pseudomonas*; Rottenberg, 1975; Thayer & Wheelis, 1982), or via a permease (as suggested by Chen *et al.*, 1984; Wong *et al.*, 1991) remains unanswered.

We have previously suggested (Wong *et al.*, 1991) that there are separate uptake systems for entry of 4-hydroxybenzoate and protocatechuate into *R. leguminosarum* and *R. trifolii*, and that these substrates gain entry into the cell via an active transport system. The major evidence for this came from the finding that ^{14}C accumulation by *R. leguminosarum* MNF300 from 4-hydroxy[^{14}C]benzoate was markedly decreased by metabolic inhibitors and by other hydroxyaromatic compounds. The detection of 4-hydroxybenzoate hydroxylase

The GenBank accession number for the sequence data reported in this paper is L23969.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Reference or source
<i>E. coli</i>		
HB101	F ⁻ <i>hsdS20</i> (<i>r_Bm_B</i> ⁻) <i>recA13 ara-14 proA2 lacY1 galK2 rpsL20</i> (Sm ^r) <i>xyl-5 mt1-1 supE44</i> ⁻	Our stock (Boyer & Roulland-Dussoix, 1969)
DH5 α	F ⁻ ϕ 80d <i>lacZ</i> Δ M15 <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (<i>r_Bm_B</i> ⁺) <i>supE44 relA1 deoR</i> Δ (<i>lacZYA-argF</i>) U169	BRL
JM83	<i>ara</i> (<i>lac-proAB</i>) <i>rpsL</i> ϕ 80d <i>lacZ</i> M15 (<i>r_km_k</i> ⁺)	Staskawicz <i>et al.</i> (1987)
MM294A	<i>pro-82 thi-1 endA1 hsdR17 supE44</i>	De Vos <i>et al.</i> (1986)
<i>R. leguminosarum</i>		
bv. <i>viciae</i>		
MNF300	Km ^S ; prototroph	Same as Johnston & Beringer (1975) strain 300
MNF3030	<i>pobA</i> ::Tn5, Km ^R ; otherwise as MNF300	Wong <i>et al.</i> (1991)
MNF3036	<i>pobA</i> ::Tn5, Km ^R ; otherwise as MNF300	Wong <i>et al.</i> (1991)
B155	B151 (pRL1JI)	Brewin <i>et al.</i> (1982)
MNF3030C1	<i>pobA</i> ::Tn5, Km ^R ; pLAFR1 + 25 kb-complementing rhizobial DNA; Tc ^R	This study
MNF3030C2	<i>pobA</i> ::Tn5, Km ^R ; pLAFR1 + 7.5 kb-complementing rhizobial DNA; Tc ^R	This study
MNF3030C3	<i>pobA</i> ::Tn5, Km ^R ; pLAFR3 + 2.0 kb-complementing rhizobial DNA; Tc ^R	This study
MNF3036C3	<i>pobA</i> ::Tn5, Km ^R ; pLAFR3 + 2.0 kb-complementing rhizobial DNA; Tc ^R	This study
<i>R. leguminosarum</i>		
bv. <i>trifolii</i>		
WU95	Km ^S ; prototroph	University of Western Australia
MNF9013	<i>pobA</i> ::Tn5-233, Km ^R ; otherwise as WU95	Wong <i>et al.</i> (1991)
MNF9010	<i>pcaHG</i> ::Tn5-233, Km ^R ; otherwise as WU95	Wong <i>et al.</i> (1991)
MNF9013C1	<i>pobA</i> ::Tn5-233, Km ^R ; pLAFR1 + 25 kb-complementing DNA; Tc ^R	This study
MNF9013C2	<i>pobA</i> ::Tn5-233, Km ^R ; pLAFR1 + 7.5 kb-complementing rhizobial DNA; Tc ^R	This study
MNF9013C3	<i>pobA</i> ::Tn5-233, Km ^R ; pLAFR3 + 2.0 kb-complementing rhizobial DNA; Tc ^R	This study
Plasmids		
pLAFR1	Broad host-range cosmid; Tc ^R	Downie <i>et al.</i> (1983)
pLAFR3	Broad host-range cosmid; Tc ^R	Staskawicz <i>et al.</i> (1987)
pUC18	Mp13 cloning vector; Ap ^R	Yanisch-Perron <i>et al.</i> (1985)
pRK2013	Mobilizing plasmid for <i>R. leguminosarum</i> ; Km ^R	Ditta <i>et al.</i> (1980)
pRK607	Mobilizing plasmid for <i>R. trifolii</i> ; Km ^R	De Vos <i>et al.</i> (1986)
pCMW3030	pLAFR1 carrying <i>EcoRI</i> fragment from <i>R. leguminosarum</i> B155 gene bank; Tc ^R	Wong <i>et al.</i> (1991)
pCMW2	pUC18 carrying Ap ^R ; Tn5 carrying Km ^R inserted into rhizobial sequences; Ap ^R , Km ^R	This study
pCMW4	Subclone of pCMW2 comprising pUC18 carrying Ap ^R and 1.2 kb of Tn5 plus flanking sequences of MNF300; Km ^S	This study
pCMW5	Subclone of pCMW11 in pLAFR3; 2.0 kb insert; Tc ^R	This study
pCMW6	Subclone of pCMW4 in pUC18; 1.05 kb insert; Ap ^R	This study
pCMW9	Subclone of pCMW4 in pUC18; 0.61 kb insert; Ap ^R	This study
pCMW11	Subclone of pCMW3030 in pLAFR3; 3.0 kb band; Tc ^R	This study
pCMW14	Subclone of pCMW11 in pUC18; 2.0 kb insert; Ap ^R	This study
pCMW15	Subclone of pCMW11 in pLAFR3; 1.45 kb insert; Tc ^R	This study
pCMW17	Subclone of pCMW11 in pUC18; 0.9 kb insert; Ap ^R	This study

Table 1 (cont.)

Strain or plasmid	Genotype or phenotype	Reference or source
pCMW18	Subclone of pCMW11 in pUC18; 1.1 kb insert; Ap ^R	This study
pCMW21	Subclone of pCMW17 in pUC18; 0.17 kb insert; Ap ^R	This study
pCMW22	Subclone of pCMW17 in pUC18; 0.72 kb insert; Ap ^R	This study
pCMW23	Subclone of pCMW18 in pUC18; 0.66 kb insert; Ap ^R	This study
pCMW24	Subclone of pCMW18 in pUC18; 0.48 kb insert; Ap ^R	This study
pCMW30	Subclone of pCMW14 in pUC18; 0.64 kb insert; Ap ^R	This study

in a mutant of *R. trifolii* WU95 (MNF9013) that could grow on protocatechuate and 4-hydroxymandelate, but not on 4-hydroxybenzoate, implied that the lesion in this mutant should be in a 4-hydroxybenzoate permease (Wong *et al.*, 1991).

The mechanism of 4-hydroxybenzoate uptake has now been re-examined by cloning and sequencing of the gene involved in uptake of this solute. This paper presents evidence that 4-hydroxybenzoate hydroxylase is required for continued entry of 4-hydroxybenzoate into cells of *R. leguminosarum*.

METHODS

Bacteria and plasmids. The bacteria and plasmids used in this study are listed in Table 1. Wild-type rhizobial strains MNF300 and WU95 are able to grow on 4-hydroxybenzoate or protocatechuate as sole carbon source. Mutants MNF3030, MNF3036 and MNF9013 are unable to grow on 4-hydroxybenzoate but can grow on protocatechuate. The protocatechuate 3,4-dioxygenase mutant MNF9010 is unable to grow on 4-hydroxybenzoate or protocatechuate.

Media. *Escherichia coli* was grown at 37 °C on 2 × YT (l⁻¹: 10 g yeast extract, 16 g tryptone and 5 g NaCl). Rhizobial strains were grown at 28 °C in minimal salts medium (MS; Brown & Dilworth, 1975) containing 40 mM HEPES, pH 7.0 (MSH), NH₄Cl (10 mM), 0.3 mM potassium phosphate and 5 or 10 mM glucose or 2 mM 4-hydroxybenzoate. Complemented and other rhizobial mutants were cultivated and maintained on media containing 12.5 µg tetracycline ml⁻¹ or 50 µg kanamycin ml⁻¹, respectively.

Plasmid mobilization and complementation. A cosmid library of *R. leguminosarum* B155 genomic DNA was kindly supplied by Dr J. A. Downie of the John Innes Institute, Norwich, UK. Plasmid transfer from *E. coli* to *Rhizobium* sp. was by triparental matings on TY medium using HB101 or J53 carrying pRK2013 or pRK607, respectively.

Isolation of chromosomal and plasmid DNA, and transformations. Total DNA was prepared from mid-exponential or stationary phase cultures of strains grown in 5 ml TY medium (supplemented with kanamycin when appropriate) by the method of Ausubel *et al.* (1988). Plasmid DNA was isolated on a CsCl gradient as described by Sambrook *et al.* (1989). Mini-preparations of plasmid DNA were extracted by the alkaline lysis method of Sambrook *et al.* (1989) and transformed into *E. coli* using the method of Hanahan *et al.* (1991).

Probe preparation. DNA probes were isolated on low-melting-temperature (LMT) agarose, purified by gelase (supplied by Epicentre) or on Magic PCR columns (Promega Corp) and

labelled with biotin or digoxigenin 11-dUTP according to the manufacturer's (Bresatec) instructions.

DNA hybridizations. Denatured, neutralized, membrane-bound DNA was added to hybridization fluid. Hybridizations were carried out at 68 °C (high stringency) and 28 °C (low stringency) and washes were as described by Sambrook *et al.* (1989).

Subcloning of complementing plasmid. Plasmid pCMW3030 was digested with *Eco*RI, religated and transformed into *E. coli* HB101. Tetracycline-resistant transformants were filter-mated separately to *R. leguminosarum* MNF3030 and MNF3036 and *R. trifolii* MNF9013 and colonies selected for growth on 4-hydroxybenzoate.

A 2.0 kb *Eco*RI-*Pst*I and a 1.45 kb internal *Pst*I contiguous fragment were subcloned into *Eco*RI-*Pst*I- or *Pst*I-cut pLAFR3 to form pCMW5 and pCMW15, respectively. These plasmids were used in complementation experiments and in further subcloning for DNA sequence analysis.

Cloning and subcloning of sequences flanking Tn5. *Eco*RI-digested genomic DNA from MNF3030 was ligated into pUC18 and transformed into *E. coli* JM83; transformants were selected on 2 × YT plus kanamycin and ampicillin. A plasmid, pCMW2 (approximately 22 kb), was isolated from two transformants and subcloned into DH5α.

Nucleotide sequence analysis. DNA sequencing was performed using the Prism Ready Reaction kit and automated sequencer from Applied Biosystems. Analysis of sequence data was performed using the MacVector computer program.

Recrystallization of hydroxymandelate. 4-Hydroxymandelate (Sigma) was dissolved in ethyl acetate, decolorized with Norit A charcoal, and crystallized by the gradual addition of an equal volume of petroleum ether.

Growth experiments. Wild-type and complemented rhizobial strains were grown with shaking at 28 °C to mid-exponential phase in MSH containing 2 mM 4-hydroxybenzoate; a similar medium containing 2 mM 4-hydroxymandelate was used to determine whether these strains could grow on 4-hydroxymandelate. The mutants were grown in similar medium but with the addition of 5 or 10 mM glucose. Cultures were harvested, washed twice in minimal salts and resuspended in 1 mM 4-hydroxybenzoate or 1 mM 4-hydroxymandelate as sole carbon source and cell density monitored.

4-Hydroxybenzoate or 4-hydroxymandelate consumption. Cells were grown essentially as described above and resuspended to an $A_{600} = 0.1-0.5$ in MSH containing 1 mM 4-hydroxybenzoate or 1 mM 4-hydroxymandelate as the sole carbon source (except where stated otherwise). Samples were removed every 1.5 or 2.0 h for measurement at A_{600} and for assay of the concentration of 4-hydroxybenzoate or 4-hydroxymandelate. The rate of substrate disappearance was

Table 2. Mean generation times for growth on 4-hydroxybenzoate, rates of consumption of 4-hydroxybenzoate and rates of transport of 4-hydroxy[14 C]benzoate for strains of *R. leguminosarum* and *R. trifolii*

The number of replicates is shown in parentheses. Replicates differed by no more than 30 %. No antibiotics were used for growth experiments.

Strain	Complementing plasmid	Mean generation time (h) on 4-hydroxybenzoate	Rates of consumption of 4-hydroxybenzoate [$\mu\text{mol h}^{-1} (A_{600})^{-1}$]	Uptake of 4-hydroxy[14 C]benzoate [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$]
MNF300	—	8.0–9.0 (4)	0.66 (2)	28.5 (3)
MNF3030	—	No growth	0	0
MNF3030C1 or MNF3030C2	pCMW3030 or pCMW11	15.0–16.0 (2)	0.37 (2)	5.5 (2)
MNF3036	—	No growth	0	0
MNF3036C1 or MNF3036C2	pCMW3030 or pCMW11	19.0–20.0 (2)	0.3 (2)	6.3 (2)
WU95	—	4.5 $\pm$ 0.5 (5)	0.88 (2)	25.5 (2)
MNF9013	—	No growth	0	0
MNF9013C1 or MNF9013C2	pCMW3030 or pCMW11	6.0–7.0 (2)	0.66 (2)	25.0 (2)

determined by plotting substrate concentration against $\int A_{600} dt$ (Dilworth *et al.*, 1983).

Uptake of 4-hydroxy[14 C]benzoate and [14 C]protocatechuate. Uptake of 4-hydroxy[7- 14 C]benzoate (Amersham) was measured as described by Wong *et al.* (1991). [14 C]Protocatechuate was prepared and its uptake measured as described by Wong *et al.* (1991).

Analytical methods. 4-Hydroxybenzoate was measured in culture supernatants by acidifying with HCl (to 0.1 M final concentration) and recording A_{255} . Concentration was calculated using an absorption coefficient of $14560 \text{ M}^{-1} \text{ cm}^{-1}$ at 255 nm. 4-Hydroxymandelate was assayed at 234 nm. Protein was determined using the method of Lowry with bovine serum albumin as a standard.

Paper chromatography. Hydroxyaromatic compounds were chromatographed and detected as described by Smith (1960).

Enzyme assays. Cell-free extracts were prepared as described by Wong *et al.* (1991) and 4-hydroxybenzoate hydroxylase was assayed using the method of Entsch *et al.* (1976). 4-Hydroxybenzoate, protocatechuate, 4-hydroxybenzaldehyde, 2,5-dihydroxybenzoic acid and carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) were supplied by Sigma.

RESULTS AND DISCUSSION

Complementation of mutants

Following conjugation with a cosmid library of *R. leguminosarum* B155 DNA in *E. coli*, one plasmid (pCMW3030) was identified which enabled MNF3030 to grow on 4-hydroxybenzoate. A 4.0 kb *EcoRI* fragment subcloned from pCMW3030 (to form pCMW11) and a 2.0 kb *EcoRI*–*PstI* subclone of it (pCMW5) enabled the mutants MNF3030, MNF3036 and MNF9013 to grow on 4-hydroxybenzoate as sole source of carbon.

Complemented mutants of *R. leguminosarum* grew slowly on 4-hydroxybenzoate, whereas the complemented mutant of *R. trifolii* was more like the wild-type (Table 2). A direct quantitative comparison of growth rates of complemented strains with those of the wild-types is not valid as a wild-type containing the cloning vector was not constructed. The rate of consumption of 4-hydroxybenzoate and growth on it appeared to be correlated in the complemented strains.

Restriction mapping

The 4.0 kb complementing DNA from *R. leguminosarum* B155 was restriction-mapped using the fragments shown in Fig. 1(a) as hybridization probes. This 4.0 kb fragment was used as a probe against *EcoRI*-digested genomic DNA from MNF300, MNF3030 and WU95. DNA from MNF300 showed two hybridizing bands (1.8 kb and 2.2 kb) and there were bands at 0.3 kb, 1.5 kb and 2.2 kb in WU95, indicating that DNA from these strains contain additional *EcoRI* sites not found in B155. DNA from the Tn5-induced mutant, MNF3030, showed bands of 2.2 kb and 7.6 kb, indicating that Tn5 had inserted into the 1.8 kb *EcoRI* fragment.

The sequences flanking Tn5 in MNF3030 were cloned and mapped (Fig. 1a). A 0.55 kb probe (see Fig. 1b) hybridized to the 1.8 kb fragment from MNF300 and the 7.6 kb fragment of MNF3030. The region of insertion of Tn5 in MNF3030 was determined using this probe against the 4.0-kb-complementing B155 DNA digested with *BglI* (Fig. 1b).

The 2.0 kb *EcoRI*–*PstI* fragment of the 4.0-kb-complementing B155 DNA (Fig. 1a) was subcloned and

Fig. 1. (a) Restriction map of the 4.0 kb fragment isolated from *R. leguminosarum* B155 able to complement mutants of *R. leguminosarum* MNF300 and *R. trifolii* WU95. There were no sites for *BclI*, *HindIII*, *HaeIII*, *KpnI*, *SacI* or *XhoI*. The isolated 4.0 kb fragment was digested with restriction enzymes (*BglI*, *HpaI*, and *PstI*) and fragments marked with an asterisk used as probes against *EcoRI* genomic DNA digests of wild-type and mutant DNAs plus the 4.0 kb fragment digested separately with *BglI* and *PstI*. The 2.0 kb *EcoRI*-*PstI* fragment was subsequently shown to also complement the Tn5 mutants. The extent of sequence data for *pobA* and *pobR* is indicated by arrows over the 2.0 kb fragment. The map is aligned with the equivalent region in MNF300. (b) Restriction map of pCMW4 isolated from the 4-hydroxybenzoate-negative mutant pCMW3030 showing IS sequences from Tn5 flanking 1.55 kb of rhizobial DNA in pUC18. The two probes (0.55 kb and 1.69 kb) marked with an asterisk were hybridized against *EcoRI*-digested genomic DNA of MNF300 and plasmids pCMW5 (1.45 kb of B155 DNA), pCMW15 (2.0 kb of B155 complementing DNA) plus the *BglI*-digested 2.0 kb complementing DNA from B155. The extent of sequence data for *pobA* and *pobR* is indicated by arrows over sequences flanking Tn5. Bg, *BglI*; B2, *BglI*; E, *EcoRI*; H, *HpaI*; P, *PstI*; Pv, *PvuI*.

found to complement the mutants. This indicated that the defective gene was located within this fragment.

DNA sequencing

The DNA sequence was determined by sequencing both strands of overlapping cloned fragments from the 2.0 kb (B155) and 1.55 kb (MNF300) fragments of DNA, using synthetic primers where appropriate.

Analysis of the sequence data revealed three ORFs comprising at least 300 nucleotides within the 2.0 kb fragment, but only one of substantial length (1.17 kb). A

second ORF (0.43 kb) is transcribed upstream and in the opposite direction to the largest. Another small ORF of 0.51 kb (transcribed in the opposite direction to the 1.17 kb ORF) is found in the centre of the major ORF but does not possess sequences necessary for transcription. The 1.17 kb ORF sequence showed substantial similarity only to the 4-hydroxybenzoate hydroxylase (*pobA*) genes of *P. aeruginosa* (Entsch *et al.*, 1988, accession M23173), *P. fluorescens* (Van Berkel *et al.*, 1992, accession L13747) and *A. calcoaceticus* (DiMarco *et al.*, 1993a, accession L03407).

The DNA sequence of the *R. leguminosarum pobA* gene from *R. leguminosarum* B155 is shown in Fig. 2(a). The gene has 1173 nucleotides (390 amino acids, beginning with leucine). The 5' end of the transcribed gene sequence was immediately preceded by the ribosome-binding sequence GGAGG, separated by six residues from the translational start. Just after the C terminus of the protein, a stem-loop structure 5' CGAGGATAATTCTGC ATCCTCG 3' was detected (Fig. 2a).

DNA from the homologous region in *R. leguminosarum* MNF300 (flanking Tn5 in pCMW4) was partially sequenced (Fig. 2b; 936 nucleotides and 312 corresponding amino acids). Tn5 had inserted 0.94 kb from the beginning of the gene. The *pobA* gene for both strains begins with a leucine codon and is preceded by the ribosome-binding sequence GGAGG.

Homology of *R. leguminosarum pobA*

About 80% of the *pobA* gene in MNF300 has been sequenced. The nucleotide sequences of *pobA* from MNF300 and B155 show 93% homology (over 936 nucleotides). The MNF300 protein differs from that from B155 by 15 residues (Fig. 3).

Comparison of the *pobA* sequences from *R. leguminosarum* B155 and *P. aeruginosa* and *P. fluorescens* (Entsch *et al.*, 1988) indicated 66% nucleotide identity and 64% amino acid identity. The *pobA* identity of *P. aeruginosa* with *R. leguminosarum* MNF300, over the 312 amino acids for which sequence has been determined, was 69% (cf 68% with B155 for the same residues). The regions associated with substrate and FAD binding are the most highly conserved (Fig. 3). The eight amino acids that have been shown to have a substrate-binding function in the *P. aeruginosa* PobA show 100% identity in both B155 and MNF300, while the 21 amino acids associated with FAD binding in *P. aeruginosa* PobA show 95% identity (Fig. 3). In particular, Tyr201 and Tyr385 (which in PobA from *P. aeruginosa* and *P. fluorescens* interact with the 4-hydroxy residue in the substrate; Van Berkel *et al.*, 1992), have been conserved in the same relative positions in *R. leguminosarum*, as have Ser212, Arg214 and Tyr222, which interact with the carboxyl moiety of the substrate.

Alignment of the derived amino acid sequences of PobA from *R. leguminosarum* and *A. calcoaceticus* revealed 60% identity (Fig. 3). The *Acinetobacter* protein (DiMarco *et al.*, 1993a) comprises 401 amino acids and appears to lack a residue somewhere between the completely conserved amino acids at position 131 (Asp) and position 137 (Pro)

(a)	
CAGCCGCCCTAACGCTCTGCATGACATAGGCTGCCTCGGCATGATCGGGATCGAGCGCGTCAGATGCGGCGCCGCCAACCATTCGCCGAGCTGGCTTCGG	148
TCGCGGGGCGGATGGCTGAGATGGGATCTCAACAGGGTGATCACCAGGCCGTCGATATCGCGTGAAAAACGAAAGCCGTGATTGAGCCGGGCGGCACGG	248
TGATGATGGCGGGCGGACGGATGGCATGGCTCTTTTCGCCGAAAAATCGCATCGCCCGAACCGCTTTTCGATGTACAATATCTGAAAGAAATCTCGTGGCG	348
GTGCGGGCGAATCTCCAGTGATGCAAACTGCTGCGAGAGCGAATTGTTTCGCAATGCACCCAAAAATCCGGTTCTCGCCCGGTCTTTTCGCCGTAGAGT	448
TCATAGGTAGGACCTGTCTGCTCATGGGGCTCCCGTGGGGCTTCGCGGACCAATGTTGATTTAGTGCATTTTAAGGTCGGCATGTCCATTGCTGCGG	548
CAAGCGCGGCACGGCAGAACTGTACAAAACGCAAAATGCAGGGAGGAGCATTTCGCAACTCAGGTCGCCATCATCGGTTCCGGGCCATCCGCCCTGCT	648
GCTCGGCCAGCTTCTGACCGAAGCCGGCATCGACAATGTCTCTCGATCGTGTGAACAAGGATTACATCCTCGGCCGGGTCGTGCCGGCGTTCTGGAG	748
GAAGGCACCGTCGGGCTGTTGGATCAGGCGCAGATCAGGCGCGAGGCTGCAATGCCGAGGGGCTGCCGCATGACGGTTTCTCGCTGGCCTTCGACGGACCGG	848
ACCATCGCATCGACCTTACGAATTGACCGGCGGCGAGGCGCTACCGCTCAGGACAGACCGAAGTGACGCGCATCTCATGGAGCGGCGCAAGAAAG	948
CGGCTCCTTATCGATCTACGATGCCGTCGACGTCGACCGCATGACTTCGACGGCCCTTCTCCCTTCGTACCTATGTGAAAGACGGTGTTCGCAAGCGC	1048
ATCGATTGTGACTTTCATCGCCGGCTGCGACGGGTTTCACGGCGCCAGCCGCAAGCCGTCGCCGAACGGGCGATCAGGAGTTTCGAGAAGATCTATCCCT	1148
TCGGCTGGCTGGGATCCTTGCCGACGTGGCGCTGTTCAGCCATGAATCATCTACGCCAACCATCCAAGGGGCTTTGCGCTCTGTTGATGCGCTCGGC	1248
CACCCGACGCGCTACTACATCCAATGTACGCTCGACGAGAAAAATCGATCACTGGAGCGACGATCGCTTCTGGACGAGTTGAGACGGCGGCTGCCGACG	1348
CATCATGCCGAGGCATTGGCGACCGCGCGCTCCTTCGAGAAATCGATTGCGCCGCTGCGCTCCTTCGTTGCCGAACCGATGCGTTTCGGCCGGCTTTTCC	1448
TGGTCGGCGACGCTGCCCATATCGTCCCGCCGACCGGGCCAAAGGATTAAACCTTGCCGCCAGCGACGTCATTACCTTTTCTCCGGGCTGATCGAGCA	1548
TTACCGCAAGGCTCGAATAGTGGCATTGACGCTTACTCGCATAAAGCGCTCGCCCGTGTGTGGAAGCCGTCGCGTTTCTCGTGGATGACGACGATG	1648
ATGCATCTTTTCCGGATACCGGTGATTTGACCGAGAAATCCAGGAGGCGGAGCTCGACTATCTCACCATTCCCGCGCCGCTCGATGGCGCTCGCGG	1748
AGAATTATGTGGGATTACCATTTCTGAGACGAGGATAATTCCTGCAATCTCGACCAATCATAAATCGCCAGGTCATGGATTACGGCATTATTTTCATTTT	1848
ACATACCATTTTCGATGACAAGCTAACGCTCAAGATTTTGGGCGGCTACCGAATACGGCGCGGGAGTGGCCAAAAAGGAGAGAGAATGAAGAAGCTA	1948
TTTCTAGCCGCGTTCGGGCGGTCGTCTCAGCGGCACGGCCTATGCAGACACCATCAAGGTCGGCGTCATCGGCCGTTACTCCGGTCCGTTCGC	2044
(b)	
GTGCGCCAGCGCTCCAAAGCTCTGCATGACATAGGCTGCCTCGGCATTTTCGCGATCGAGCGCGTCAGATGCGGCGTAGCAAACCATTCGCCGAGATGG	103
CTTCGCTCGCCGGGCGGATGGCTGAGATGGGATCTCAACAGGGTGATCACCAGGCCGTCGATATCGCGTGAAAAACGAAAGCCGTGATTGAGACCGGGCG	203
GCACAGTGATGATGGCCGGCGGACGGATGGCGTGACTTTTTTCGCCGAAGATCGCATCACCCGAACCGCTTCAATGTACAATATCTGAAAGAAATCTC	303
GTGACGGTGGCGACGAATCTCCCATTTGATGCAAGCTGCTGCGAGAGCGAATTGTTTCGCAATGCACCCAAAAATCCGGTTCTCGCCCGGTCTTTTCGCCG	403
TAGAGTTCATAGGTAGGACCTGTCTGCTCATGGGGTTCGCTGGGGTTTCTTCGGAATCAATGTTGATTTAGTGCATTTTATAAGGTTGGGATGTCCATTG	503
TTGCGGCAAGCGGACACGGCAGAACTGTGAAAAACGCAAAATGCAGGGAGGAGCATTTCGCAACTCAGGTCGCCATCATCGGTTCCGGACCCCTCCG	603
GCCTGTTGTTTCGGCCAGCATCTGACCGAAGCCGGCATCGAGAATGTCTCTCGATCGTGTGAACAAGGATTACATCCTCGGCCGGGTCGTGCCGGCGCT	703
TCTGGAGGAAGCACCGTTGGGCTGCTGGATCAGGCAAAAGCCGGGACGCGGCTACATGCCGAGGGCCTGCCGCATGACGGTTTCTCATTTGGCCTTCGAC	803
GGACGCGACCATCGCATTTGACCTCCACGAATTGACCGGCGGACGCGCTGACGGTTTATGGACAGACCGAAGTGACGCGCATCTCATGGAGCGCGTG	903
AAGCGAGCGGCTCCCTGTGATCTACGATGCGGTGATGTGCGCGCGCATGATTTTCGACGGCCACTCTCCCTTCGTACCTATGTGAAAGATGGTGTGTC	1003
CAAGCGCATCGATTGCGACTTCATCGCCGGCTGCGACGGGTTTCACGGGCTCAGCGCGCAAGGCCGTCGCCGAACGGGCGATCAGGAGTTTCGAGAAGGTC	1103
TATCCCTTCGGCTGGCTGGGGTCTTTCGCGACGTGGCGCTGTCTAGTCAATGAATGATCTACGCCAACCATCCAAGGGGCTTTGCGCTCTGTTTCGATGC	1203
GCTCGGCGACCCGACGCGCTACTACATCCAATGCGGCTCGACGAGAAATCGAGAACTGGAGCGACGATCGCTTCTGGGACGAGTTGAGACGGCGTCT	1303
GCCGCTGATCACGCCGAGGCTTTCGCGACCGCGCGCTCCTTCGAGAAATCGATCGCGCGCTGCGCTCCTTCGTCGCCGAACCGATGCGTTTCGGCCGG	1403
CTTTTCTGGTGGCGACGCGCCCATATCGTCCCGCCGACCGCGCAAGGGACTGAACCTTGCCGCCAGCGACGTTTCTATCTTTTCTCCGGGCTG	1503
ACTCTTATACAAAGGACTG	
Tn5	

Fig. 2. (a, b). For legend see facing page.

present in both *R. leguminosarum* strains and in the *Pseudomonas* spp. Additional residues are found at the C terminus of the *Acinetobacter* protein. Again, regions associated with substrate and FAD binding are highly conserved.

Both the *Pseudomonas* PobA proteins comprise 394 amino acids (Entsch *et al.*, 1988; Van Berkel *et al.*, 1992) compared with 390 in the *R. leguminosarum* PobA; the four extra residues in the *Pseudomonas* enzyme are found at the C terminus.

As in *Pseudomonas* (Entsch *et al.*, 1988), there is a high G+C content in the coding portion of the *R. leguminosarum* gene (61% vs 68% in *Pseudomonas*) and the codons are dominated by C and G in the third (degenerate) position (75% in *R. leguminosarum* vs 90% in *P. aeruginosa*). The preference for G and C in the first (codon) position in the *P. aeruginosa* *pobA* (69%) also occurs in the *R. leguminosarum* gene (63%).

Whilst there is identity of more than 99% between the PobA amino acid sequences in different species of *Pseudomonas* (*P. aeruginosa* and *P. fluorescens*), identity of these sequences between *Rhizobium* strains (B155 and MNF300) is less (95% for 80% of the coding portion of the gene). More variation was revealed when a probe of 0.17 kb isolated from the *R. leguminosarum* B155 *pobA* gene (in a region associated with substrate binding in *Pseudomonas*) was hybridized against genomic DNA from other strains and species of *Rhizobium*. Other strains of *R. leguminosarum* bv. *viciae* (WSM710, SU391) showed a band of the same (1.8 kb) or similar size to that in MNF300 (although 710 showed a single fragment of 3.8 kb), but different strains of *Rhizobium* (including *R. trifolii*, *R. phaseoli* and *R. meliloti*) and other species (*P. aeruginosa*) revealed genomic *EcoRI* fragment(s) sizes ranging from 0.3 kb to 6.7 kb.

Promoter and operator regions of *pobA* in *R. leguminosarum*

The similarities between *pobA* from *R. leguminosarum* and *P. aeruginosa* do not extend to the promoter and operator regions. Whilst the consensus ribosome-binding site (RBS) in *P. aeruginosa*, 5' GAGG 3' found 10 bp upstream from the start codon (Entsch *et al.*, 1988), is similar to that found in *R. leguminosarum*, the operator palindrome sequence (of 21 bp) found 29 bp upstream from the *P.*

aeruginosa start codon is not found in *R. leguminosarum*. No palindromic sequences are found in this region in either *R. leguminosarum* B155 or MNF300.

A feature of the regulatory region of *pobA* in *P. aeruginosa* is the occurrence of A+T-rich regions in a gene which overall is 68% G+C. In *R. leguminosarum* (B155 and MNF300) the regions upstream of the start codon also contain regions which are A+T-rich in comparison with the *pobA* gene. Specifically, the sequence 5' AATTTT 3' (in B155) and 5' TTTATA 3' (in MNF300) occur in the same position upstream of the start codon as the -10 site in *P. aeruginosa* (Fig. 2a and b). Both of these putative -10 sites occur in the regulatory region at the richest A+T sites. Similarly, the putative -35 sites in *R. leguminosarum*, 5' CCGACA 3' (B155) and 5' GGAATC 3' (MNF300), are found 18 bases upstream from their respective -10 sites (Fig. 2a and b). Neither of the proposed -10 and -35 sites (nor any regions nearby) show similarity with promoter sites for other *Rhizobium* genes (Gray *et al.*, 1990; Bae & Stauffer, 1991; De Maagd *et al.*, 1992). Sequences found in similar positions upstream of the start codon for *pobA* in *A. calcoaceticus* (DiMarco *et al.*, 1993b) do, however, show similarity to these putative promoter sites in *R. leguminosarum*. The sequence 5' AATTTT 3' occurs in the same (-10) region in *A. calcoaceticus*.

The -35 site in *A. calcoaceticus*, 5' GACAGG 3' (DiMarco *et al.*, 1993b), shows little similarity to the putative -35 sites in *R. leguminosarum* B155 and MNF300, although the B155 site does show three matches of six to the *E. coli* σ^{70} consensus sequence 5' TTGACA 3' (Harley *et al.*, 1987). Sequence dissimilarity in the -35 region is not uncommon in positively regulated promoters (Raibaud & Schwartz, 1984).

The putative -10 site for *R. leguminosarum* MNF300 (5' TTTATA 3') is identical to part of a possible integration host factor (IHF)-binding site proposed for a RopA (repressed outer membrane) protein of *R. leguminosarum* (De Maagd *et al.*, 1992).

Regulation of *pobA* in *R. leguminosarum*

A transcriptional activator (*pobR*) was recently described for *pobA* in *A. calcoaceticus* (DiMarco *et al.*, 1993b). Inducible expression of cloned *pobA* in *E. coli* depended on the presence of a functional *pobR*. Regions upstream of the *R. leguminosarum* *pobA* (B155 and MNF300) were

Fig. 2. (a) Sequence data obtained for the 2.0 kb-complementing DNA from *R. leguminosarum* B155 comprising the gene for 4-hydroxybenzoate hydroxylase (*pobA*) and its putative positive transcriptional regulator (*pobR*). The sequence for *pobA* in the 5'-3' direction begins at nucleotide 602. The sequence for *pobR* is shown in the 3'-5' orientation with the initiator codon for the amino-terminal amino acid of the protein beginning at nucleotide 474. The last digit of each number identifies the exact nt which corresponds to that number. The nucleotide sequences highlighted above 'terminator' delineate a palindromic sequence associated with termination of transcription of *pobA*. The sequences above RBS correspond with those attributed to the ribosome-binding sites. Putative promoter regions -10 and -35 upstream of the *pobA* RBS are also highlighted. (b) Partial nucleotide sequence for the *pobA/pobR* region from MNF300. The sequence for *pobA* in the 5'-3' direction begins at nucleotide 563 and extends to the site of insertion of Tn5 (highlighted). The sequence for *pobR* is shown in the 3'-5' orientation with the initiator codon for the amino-terminal amino acid of the protein beginning at nucleotide 435. The sequence above RBS corresponds with that attributed to the ribosome-binding site for *pobA*. Putative promoter regions -10 and -35 upstream of the *pobA* RBS are also highlighted.

Fig. 3. Identity between amino acid sequences of *pobA*. Boxed regions indicate sequences where three or more of these strains are identical. The gap introduced into the amino acid sequence of *A. calcoaceticus* for the purposes of alignment is indicated by a dash. 1, *R. leguminosarum* B155; 2, *R. leguminosarum* MNF300; 3, *P. aeruginosa* PAO1C (Entsch et al., 1988); 4, *A. calcoaceticus* ADP1 (DiMarco et al., 1993a); *, amino acids which are important for FAD binding in *Pseudomonas*; #, amino acids which are important for substrate binding in *Pseudomonas*.

examined to determine whether a regulatory *pobR* gene exists in *Rhizobium*. ORFs of 0.43 kb were found on the complementary strand of both the 2.0 kb *R. leguminosarum*

B155-complementing fragment and the sequences flanking Tn5 in MNF300, extending through to the end of each of the fragments (Fig. 1a and b).

Fig. 4. Similarity allowed by MacVector protein analysis software between amino-terminal amino acid sequences of *pobR*. A small number of amino acids have been included from the ORF found at the beginning of sequence data for the *P. aeruginosa pobA* (Entsch *et al.*, 1988). The sequences underlined are those proposed for the helix-turn-helix motif in *A. calcoaceticus* (DiMarco *et al.*, 1993b). 1, Amino acid sequence of *pobR* for *R. leguminosarum*; 2, amino acid sequence of *pobR* for *A. calcoaceticus*; 3, amino acid sequence of *pobR* for *P. aeruginosa*.

Regions of similarity of the B155 ORF to the *A. calcoaceticus pobR* are shown in Fig. 4. The consensus RBS sequence 5' GGAG 3' is separated from the translational start by 4 nucleotides (cf GGAGG separated by 4 nucleotides from the start codon of *catR* in *A. calcoaceticus*; Rothmel *et al.*, 1990). As in *A. calcoaceticus*, the *pobA* and *pobR* genes appear to be transcribed divergently in B155 and MNF300, the separation being 130 bp and 128 bp, respectively. The sequenced portion of the *pobR* gene (Fig. 4) contains information for a 142 amino acid segment of the amino-terminal portion of the PobR protein. In *A. calcoaceticus*, PobR contains 271 amino acids; if the protein is of a similar size in *R. leguminosarum*, this would suggest that about half the *pobR* gene has been sequenced. The 142 amino acid amino-terminal segment of PobR in *R. leguminosarum* has a very high ratio (5.5:1) of arginine to lysine, analogous to *A. calcoaceticus* (DiMarco *et al.*, 1988) and the rare arginine codon AGA is used twice in the corresponding regions of the genes in both organisms. These similarities to the *A. calcoaceticus pobR* are evidence that these sequences are part of *pobR* in *R. leguminosarum*. Comparison of the sections of *pobR* obtained for B155 and MNF300 shows nucleotide homology of 95 % and amino acid identity of 94 %.

The role of *pobA* in the uptake of 4-hydroxybenzoate

The demonstration that the gene mutated in MNF3030, MNF3036 and MNF9013 codes for 4-hydroxybenzoate hydroxylase suggests that this enzyme is involved in the

uptake of 4-hydroxybenzoate. Previous work (Wong *et al.*, 1991) had suggested, on the basis of inhibitor and substrate analogue studies, that 4-hydroxybenzoate uptake was active. We have now re-examined the mechanism of uptake of 4-hydroxybenzoate using the complemented strains.

Assays of 4-hydroxybenzoate hydroxylase in wild-type and mutants

In the earlier work (Wong *et al.*, 1991), extracts from MNF9013 showed 4-hydroxybenzoate hydroxylase activity essentially the same as for the wild-type. Assays of hydroxylase activity (Table 3) in two of the complemented mutants (MNF3030C3 and MNF9013C3) carrying the 2.0 kb complementing fragment showed that they had enzyme activities which were comparable to that for the fully-induced wild-type MNF300 ($37 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$). Enzyme assays were not carried out on complemented mutants carrying the larger plasmids (MNF3030C1, MNF3030C2, MNF9013C1 and MNF9013C2). None of the mutants showed significant hydroxylase activity (Table 3). Hydroxylase activity in MNF9010, a protocatechuate 3,4-dioxygenase (*pcaHG*) mutant (Wong *et al.*, 1991) grown on 4-hydroxybenzoate (2 mM) and glucose (10 mM) was also measured to assess the degree of repression exerted by glucose when mutants such as MNF3030 and MNF9013 were grown in media containing glucose and 4-hydroxybenzoate. The hydroxylase activity in MNF9010 was 17% of that found for the fully-induced wild-type, a rate comparable to that expected

Table 3. Rates of hydroxylase activity in *R. leguminosarum pobA* mutant MNF3030 and complemented *pobA* mutant MNF3030C3, and in *R. trifolii pobA* mutant MNF9013, complemented *pobA* mutant MNF9013C3 and *pcaHG* mutant MNF9010

The number of replicates is shown in parentheses. Replicates differed by no more than 20 %.

Strain	Carbon source(s) for growth	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]
MNF300	4-Hydroxybenzoate	37.0 (2)
MNF3030	Glucose + 4-hydroxybenzoate	0.03 (2)
MNF3030C3	4-Hydroxybenzoate	49.1 (2)
MNF9013	Glucose + 4-hydroxybenzoate	0.67 (2)
MNF9013C3	4-Hydroxybenzoate	30.6 (2)
MNF9010	Glucose + 4-hydroxybenzoate	6.6 (2)

when the pathway is repressed by glucose (Dilworth *et al.*, 1983; Wong *et al.*, 1991).

Effects of other compounds on hydroxylase activity

When cells of the prototroph MNF300 grown on 4-hydroxybenzoate were incubated with a 50-fold molar excess of protocatechuate (5 mM), accumulation of 4-hydroxy[¹⁴C]benzoate was totally inhibited (Wong *et al.*, 1991); similar experiments with 4-hydroxybenzaldehyde (2 mM) showed a 100 % inhibition of 4-hydroxy[¹⁴C]benzoate accumulation whereas 2,5-dihydroxybenzoate (2 mM) caused only 29 % inhibition. We had interpreted these data as implying competition for a transport system; an alternative explanation is that these compounds interfere with 4-hydroxybenzoate hydroxylase activity intracellularly.

In hydroxylase assays containing 0.3 mM 4-hydroxybenzoate, 0.1 mM protocatechuate inhibited hydroxylase activity by 93 %; 0.6 mM 4-hydroxybenzoate was able to overcome this inhibition, but not when protocatechuate was present at 1 mM. 4-Hydroxybenzaldehyde (1 mM) inhibited enzyme activity by 76 %, but 2,5-dihydroxybenzoic acid at 2 mM inhibited hydroxylase activity by only 17 % and by 23 % at 4 mM. These results are comparable with the degree of apparent inhibition of labelled 4-hydroxybenzoate accumulation by these hydroxyaromatic compounds.

Accumulation of ¹⁴C from 4-hydroxy[¹⁴C]benzoate and [¹⁴C]protocatechuate in MNF300 was inhibited over 98 % by 0.05 mM CCCP (Wong *et al.*, 1991), apparently indicating inhibition of an energized uptake system. However, when the enzyme reaction was allowed to proceed for 1 min [at 37 nmol min⁻¹ (mg protein)⁻¹]

prior to the addition of 0.025 mM CCCP, enzyme activity fell to 17.4 nmol min⁻¹ (mg protein)⁻¹. Addition of a further 0.025 mM CCCP after 2 min lowered activity to 9.1 nmol min⁻¹ (mg protein)⁻¹; a further 0.1 mM added after 5 min resulted in a final rate of 1.5 nmol min⁻¹ (mg protein)⁻¹ (96 % inhibition).

Thus, the apparent effects of both hydroxyaromatic compounds and of metabolic inhibitors such as CCCP (Wong *et al.*, 1991) on [¹⁴C]hydroxybenzoate accumulation may be explained by these compounds inhibiting 4-hydroxybenzoate hydroxylase activity and preventing continued entry of labelled substrate into the cells.

Growth on 4-hydroxymandelate

Wong *et al.* (1991) reported very slow growth of both *R. trifolii* WU95 and MNF9013 on 4-hydroxymandelate in liquid media. A lesion in *pobA* should not allow growth on 4-hydroxymandelate as sole carbon source because 4-hydroxymandelate must be converted to 4-hydroxybenzoate to be metabolized. Paper chromatography of the original 4-hydroxymandelate failed to reveal any significant contamination. When this material was recrystallized, neither the wild-type strains (MNF300, WU95) nor the mutants (MNF3030, MNF3036, MNF9013) grew on 4-hydroxymandelate. Apparent growth of WU95 and MNF9013 on 4-hydroxymandelate in earlier work may have been due to microbial contamination or contamination of the medium with a carbon substrate other than 4-hydroxymandelate.

Mechanism of uptake of 4-hydroxybenzoate and protocatechuate by *R. leguminosarum*

Independently isolated mutants of *R. leguminosarum* MNF300 (MNF3030 and MNF3036) and *R. trifolii* WU95 (MNF9013) unable to grow on 4-hydroxybenzoate regained this ability when they carried a 2.0 kb fragment of *R. leguminosarum* B155 DNA carrying only the *pobA* gene. When *pobA* carries a Tn5 or Tn5-233 insertion, cells of *R. leguminosarum* or *R. trifolii* do not accumulate radioactive 4-hydroxybenzoate (Table 2), implying that this gene is essential for the continued entry of 4-hydroxybenzoate. If these organisms also possessed an active 4-hydroxybenzoate permease system, it would be logical to expect 4-hydroxybenzoate accumulation to occur in mutants lacking the hydroxylase; this was not observed.

Whatever mechanism is suggested for the entry of 4-hydroxybenzoate into rhizobial cells must be consistent with the observed rate of degradation of this substrate. Previous work described the stoichiometric conversion of 4-hydroxybenzoate to protocatechuate by a *pcaHG* mutant (MNF9010) of *R. trifolii* WU95 which, unable to further metabolize protocatechuate, secreted it into the medium (Wong *et al.*, 1991). The rate of this conversion, ca 28 nmol min⁻¹ (mg protein)⁻¹, gives an estimate of the rate of entry of 4-hydroxybenzoate into the cell consistent with the observed rate of uptake of 4-

hydroxy[¹⁴C]benzoate by MNF300 and with the observed activity of 4-hydroxybenzoate hydroxylase (Table 3).

Previous work has also suggested that separate systems exist for the uptake of 4-hydroxybenzoate and protocatechuate in the two biovars of *R. leguminosarum* (Wong *et al.*, 1991). MNF3030, MNF3036 and MNF9013, which were unable to accumulate 4-hydroxy[¹⁴C]benzoate, grew on protocatechuate as sole carbon source and appeared (in 30 min uptake assays) to accumulate [¹⁴C]protocatechuate. If uptake of 4-hydroxybenzoate is diffusive, the question arises as to whether uptake of protocatechuate is active or also occurs by diffusion. Short term (5 min) uptake experiments with the protocatechuate dioxygenase (*pcaHG*) mutant of *R. trifolii*, MNF9010, did not show accumulation of [¹⁴C]protocatechuate. The apparent accumulation of [¹⁴C]protocatechuate by MNF9010 over 30 min (Wong *et al.*, 1991) may therefore indicate non-specific binding of [¹⁴C]protocatechuate to the cell surface over this period of time rather than uptake by the cell.

In the model we are proposing, 4-hydroxybenzoate is 'pulled' across the membrane as a result of its intracellular conversion to protocatechuate. Uptake of 4-hydroxybenzoate would therefore be via a passive mechanism dependent upon its metabolism. The existence of a lipid-soluble carrier for 4-hydroxybenzoate in the membrane of the cell is a possibility yet to be explored.

In addition to inducing catabolic genes, phenolic compounds typical of plants also induce genes required to form active associations with host plants. Thus, the formation of nodule-initiating oligosaccharides by rhizobia is induced by flavonoids exuded from growing legume roots (Peters & Verma, 1990). To be effective at sites of transcription, these phenolic compounds must cross the cell membrane. It has been proposed that the *R. leguminosarum nodD* gene inducer, naringenin, traverses the outer membrane through aqueous pores and accumulates in the cytoplasmic membrane (Recourt *et al.*, 1989). The mechanism for the diffusion of 4-hydroxybenzoate is different; it must also pass into the cytoplasm for its subsequent catabolism to protocatechuate.

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