Elucidation of the Flavonoid Catabolism Pathway in *Pseudomonas putida* PML2 by Comparative Metabolic Profiling

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Flavonoids are 15-carbon plant secondary metabolites exuded in the rhizosphere that hosts several flavonoid-degrading bacteria. We studied flavonoid catabolism in a plant growth-promoting rhizobacterial strain of *Pseudomonas* by using a combination of biochemical and genetic approaches. Transposants carrying mini-Tn5gfp insertions were screened for flavonoid auxotrophy, and these mutant strains were found to be unable to grow in the flavonols naringenin and quercetin, while their growth in glycerol was comparable to that of the parental strain. In order to understand flavonoid catabolism, culture supernatants, whole-cell fractions, cell lysate, and cell debris of the wild-type and mutant strains were analyzed. Intermediates that accumulated intracellularly and those secreted in the medium were identified by a combination of reversed-phase high-pressure liquid chromatography and electrospray ionization-mass spectrometry. Structures of four key intermediates were confirmed by one-dimensional nuclear magnetic resonance spectroscopy. Comparative metabolic profiling of the compounds in the wild-type and mutant strains allowed us to understand the degradation events and to identify six metabolic intermediates. The first step in the pathway involves 3,3'-dihydroxylation, followed by hydrolysis and cleavage of the C-ring, leading via subsequent oxidations to the formation of protocatechuate. This is the first report on quercetin dehydroxylation in aerobic conditions leading to naringenin accumulation.

A diverse group of compounds of plant origin are ubiquitously exuded into the rhizosphere by roots. Such compounds are known to affect microbial growth by serving as nutrient sources, through their antimicrobial activities, or by directly affecting microbial gene expression (12, 13, 16, 18, 31, 34, 36, 42, 51, 53). Among the various root exudates, the most diverse classes of signal molecules are the plant secondary metabolites. Efficient utilization of these metabolites can lead to a positive selection of the utilizers. It is, therefore, not surprising that several secondary metabolite degraders have been reported (8, 22, 23, 26, 39, 41, 52, 55). Flavonoids belong to a class of 15-carbon secondary metabolites that have a three-ringed structure and are mostly found conjugated with glycosides. Due to their rich carbon content, flavonoids have potential significance as nutritional sources, whereas their complex structures allow them to have diverse pharmacological and physiological effects (30).

Several microbes, such as *Rhizobia*, *Agrobacterium*, *Pseudomonas*, *Bacillus*, and *Rhodococcus* spp., that exist in the rhizosphere are known to participate in the breakdown or degradation of flavonoids (2, 3). Although, some mechanisms have been proposed, the pathways were not elucidated. A survey of flavonoid-degrading rhizobial strains revealed that flavonoids were generally cleaved via C-ring fission (38, 39). In particular, there is a dearth of information on the biochemical and genetic aspects of catabolism of flavonoids in *Pseudomonas* spp. An early study on flavonoid degradation by a soil pseudomonad speculated on the presence of oxygenases based on the oxida-

tion products and proposed that the degradation proceeded via protocatechuate production (46). The anaerobic degradation of flavonoids by the intestinal microflora, in comparison to the aerobic pathways, has been well documented, and several reports describe the reduction and dehydroxylation reactions leading to phloroglucinol formation (10, 15, 26, 43, 44, 54, 55).

This study was undertaken to elucidate the pathway for utilization of the archetypal flavonoid, quercetin, in a plant growth-promoting rhizobacterial (PGPR) strain of *Pseudomonas putida* which can also grow on naringenin, daidzein, apigenin, hesperetin, naringin, protocatechuate, *p*-hydroxy benzaldehyde, or *p*-hydroxy benzoic acid as the sole carbon source. In order to understand the catabolism of quercetin in *Pseudomonas*, we constructed several transposon insertion mutants defective in flavonoid catabolism and compared the metabolic profiles of pathway intermediates in the cultures of wild-type and three mutant strains by using high-pressure liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy. We report for the first time the conversion of quercetin to naringenin by a soil pseudomonad involving dehydroxylation in aerobic conditions. Subsequent events such as hydrolysis and oxidation lead to formation of protocatechuate that is channelled into the tricarboxylic acid (TCA) cycle via the meta-cleavage pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids. Properties of bacterial strains used in this study are summarized in Table 1. *Escherichia coli* cultures were grown at 37°C in Luria-Bertani medium. All *P. putida* cultures were grown at 30°C in Stainer's minimal mineral medium (SM) (48) supplemented with carbon sources as described here. Based on the appropriate flavonoid levels given here, equivalent numbers of moles of carbon for glycerol were supplied in all media used in the present study. When required, the antibiotics chloramphenicol, gentamicin,

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TABLE 1. Bacterial strains used in this investigation and their properties

Strain or plasmid	Genotype or phenotype ^a	Source or reference
Strains		
<i>P. putida</i> PML2	Spontaneous Cm ^r mutant of a PGPR ATCC 39270 strain; utilizes quercetin, naringenin, daidzein, apigenin, hesperetin, and naringin	This study
<i>Flav</i> 1-9	mini-Tn5gfp mutants of PML2; quercetin negative, naringenin negative; Cm ^r Gm ^r Km ^r	This study
<i>E. coli</i> SM10 (λpir)	(<i>pro thi-1 thr-1 leuB6 supE44 tonA21 hsdR⁻M⁺ lacY1 recA</i> RP4-2-Tc:Mu-Km, λpir), a derivative of <i>E. coli</i> C600, conjugative donor of mini-Tn5	49
Plasmid (pAG408)	Carries mini-Tn5gfp, Km ^r , Gm ^r , Ap ^r , <i>tnp</i> , RP4 <i>oriT</i> , R6K <i>oriV</i>	49

^a Ap^r, Cm^r, Gm^r, and Km^r, ampicillin, chloramphenicol, gentamicin, and kanamycin resistance, respectively.

kanamycin, and ampicillin (Sigma Chemical Co., St. Louis, Mo.) were added to final concentrations of 30, 10, 15, and 100 µg/ml, respectively.

Chemicals and reagents. HPLC-grade acetonitrile and methanol (J. T. Baker), were used for all the experiments. Analytical reagent-grade hydrochloric acid, glacial acetic acid, and sodium hydroxide were from Amersham Pharmacia Biotech (Uppsala, Sweden). The flavonoids quercetin (3,3',4',5,7-pentahydroxyflavone) and naringenin (4',5,7-trihydroxyflavanone) (Fig. 1) and other aromatic metabolites such as 1,3,5-trihydroxy benzene (phloroglucinol), 3,4-dihydroxy benzoic acid (protocatechuic acid), *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoic acid were obtained from Sigma. 1,2,3-Trihydroxy benzene (pyrogallol), 3,4-dihydroxyphenylacetic acid (homoprotocatechuic acid), 3-hydroxyphenylacetic acid, 4-phenylacetic acid (α-toluic acid), 3,4-dihydroxybenzaldehyde, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid), 3-hydroxybenzoic acid, 3-(4-hydroxyphenyl)propionic acid (phloretinic acid), 4-methyl-1,2-benzenediol (4-methyl catechol), 3-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid (caffeic acid), and 2,4-dihydroxybenzoic acid were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Flavonoid stock solutions (10 mM) were prepared in alkalized methanol under liquid nitrogen conditions and filter sterilized prior to addition to media. All flavonoid solutions were stored in the dark at 4°C to prevent photosensitive reactions from occurring. Pure water (18 mΩ resistance) was used

throughout the experiments. Deuterated dimethyl sulfoxide (DMSO; 99.9%) was obtained from Aldrich and used as the solvent in NMR analysis. Deuterated water was used to blank water peaks, if any.

Mini-Tn5gfp mutagenesis and screening. Mutant strains were generated by conjugating pAG408::mini-Tn5gfp from *E. coli* SM10 (λpir) into *P. putida* strain PML2. Mutagenesis with mini-Tn5gfp was achieved by mixing 4×10^7 *Escherichia coli* SM10 (λpir) donor cells and 10^7 recipient cells of *P. putida* strain PML2 on antibiotic-free tryptic soy agar plates. After 5 h of incubation at 30°C, the cells were harvested and suspended in 0.8% saline, and subsequent dilutions were plated on SM (48) containing 0.5% glycerol as the carbon source, along with the antibiotics chloramphenicol and gentamicin as indicated. Colonies emerging on this medium were screened by replica plating them for auxotrophy to 10 mM flavonoids (quercetin). Mutant strains with the inability to grow on SM plates containing 10 mM flavonoids as the carbon source were chosen for further experiments. In order to calculate the rate of spontaneous mutations, 10^8 cells of *P. putida* PML2 (wild type) were spread on plates containing 10 mM flavonoids, and those not able to grow were assumed to be spontaneous mutants.

Growth experiments and depletion studies. Growth studies of PGPR *P. putida* PML2 wild-type and mutant strains were done in SM supplemented with 10 mM flavonoids (quercetin and naringenin; Fig. 1) as the carbon source (SMF). Cultures grown to an A_{600} of 0.2 in SM supplemented with glycerol (SMG) were diluted 10-fold for inoculating SMF. Growth was monitored by measuring the absorbance at 600 nm. Growth in other aromatic metabolites, such as naringenin, 3,4-dihydroxycinnamic acid, phloroglucinol, protocatechuic acid, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoic acid (Fig. 2) at 10 mM concentrations was studied as described above. Colony counts from plating appropriate serial dilutions were performed to further confirm the growth of wild-type and mutant strains in SMF. All counts were performed at least in triplicate, and the experiments were performed three times. Statistical analyses were performed on all data. The mean and standard error results of these values were calculated after log₁₀ transformations.

For depletion studies, samples were periodically collected from culture supernatants and assayed for flavonoid levels. Samples were filter sterilized by using a 0.22-µm-pore-size polyethersulfone membrane filter (Millipore, Bedford, Mass.) and analyzed by MS. Peak area integral values in comparison to commercially available standards were used to quantify flavonoid depletion.

Confirmation of mini-Tn5gfp insertions. Chromosomal DNA was prepared as previously described (50), and standard recombinant DNA techniques were used according to the method of Sambrook et al. (40). PCR was carried out with transposon-specific primers to confirm the presence of transposon DNA in the mutant strains. The primer sequences were derived from mini-Tn5gfp sequence (49) for the *gfp* and gentamicin resistance (Gm^r) genes (GenBank accession numbers U62636 and P23181, respectively) and were as follows: GfpF, 5'-GGA GAAGAACTTTTCACTGGAG-3'; GfpR, 5'-TTGTAGAGCTCATCCATGC CAT-3'; GmF, 5'-AGGTGGCGGTACTTGGGTC-3'; and GmR, 5'-AGAACC TTGACCGAACGC-3'. Southern blotting was performed to characterize the transposon mutants. Genomic DNAs from *P. putida* strain PML2 and its mutant strains were digested with the restriction enzyme *Bgl*II or *Kpn*I (Fermentas AB, Vilnius, Lithuania). Transfer of DNA to a nylon membrane (Hybond+; Amersham Pharmacia Biotech UK, Ltd.) from agarose gels and hybridization conditions were in accordance with the manufacturer's recommendations. Hybridization was done by using a digoxigenin-labeled PCR fragment of the transposon as the probe and visualized with nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-

FIG. 1. Structures of parent compounds used in screening for flavonoid auxotrophy in this study. (A) 3,3',4',5,7-Pentahydroxy flavone (quercetin dihydrate) (molecular weight, 338.4). (B) 4',5,7-Trihydroxyflavanone (naringenin) (molecular weight, 272.3).

3, 4-dihydroxycinnamic acid (caffeic acid), MW: 180.16

4-hydroxyphenylacetic acid, MW: 152.15

3, 4-dihydroxyphenylacetaldehyde, MW: 152.15

4-hydroxy-3-methoxyphenyl acetic acid (homovanillic acid), MW: 182.18

Isoamyl cinnamate, MW: 218.29

1, 2, 3-trihydroxybenzene (pyrogallol), MW: 126.11

2, 5-dihydroxybenzoic acid, MW: 154.12

2, 4-dihydroxybenzoic acid, MW: 154.12

3, 4-dihydroxybenzoic acid (protocatechuic acid), MW: 154.12

1, 3, 5-trihydroxybenzene (phloroglucinol), MW: 126.11

3, 4-dihydroxyphenyl propionic acid, MW: 182.18

FIG. 2. Structures of chemicals and intermediates used in this study. MW, molecular weight.

indolyolphosphate) and CSPD (Boehringer Mannheim, GmbH, Mannheim, Germany), as recommended by the manufacturer.

Flavonoid degradation experiments. The degradation experiments were performed with 200-ml cultures grown in 500-ml Erlenmeyer flasks. These flasks were inoculated with a 10-fold dilution of an overnight-grown culture adjusted to an A_{600} of 0.2 wrapped in aluminum foil, and incubated at 30°C on a rotary shaker (220 rpm). Samples were collected periodically and initially centrifuged at $16,800 \times g$ for 20 min at 4°C, and the whole-cell supernatant was separated. The pelleted cells were lysed by using a SIM-Aminco French Pressure Cell (Spectronic Instruments, Inc., Rochester, N.Y.) at 1,000 lb/in² in 0.5 M phosphate buffer. The resulting crude cell lysate was further centrifuged at $16,800 \times g$ for 20 min at 4°C to separate the cell debris from the cleared lysate. The whole-cell supernatant, crude debris fraction, and cleared cell lysate were thus collected and stored at -80°C in the dark till further use. Frozen samples of these fractions were used for the analysis of substrates and degradation products. Prior to experiments, methanol was added to 50% (vol/vol) to the samples to achieve efficient extraction of the phenolic flavonoids and their respective intermediates. Extracts were filtered with a 0.45- μ m-pore-size polytetrafluoroethylene membrane filter (Nalge Co., Rochester, N.Y.), and aliquots of 1.5 to 2.0 ml were subjected to reversed-phase (RP)-HPLC to detect the presence of various breakdown products. Uninoculated control flasks were used to identify spontaneous changes in the supplemented metabolites.

RP-HPLC analysis. Chromatographic analysis was carried out with a Vision Bio-Cad RP-HPLC workstation BIOCAD 700E equipped with a dual-pump system (PerSeptive Biosystems, Foster City, Calif.), a diode-array detector UVD-

320, and a Hypersil C₁₈ RP column (250 by 4.6 mm) with a particle size of 5 μ m (catalog no. 00G-4053-E0; Phenomenex). The column temperature was maintained at 25°C. Sample volume was kept constant at 1.5 ml for each injection, while the solvent flow rate was maintained at 0.60 ml/min. For all compounds, a gradient was used starting with HPLC-grade water (pH 3.0 adjusted with glacial acetic acid), with an increasing concentration of HPLC-grade 100% acetonitrile (a linear gradient of 1 to 10% for 4 min, a linear gradient of 10 to 60% for 60 min, followed by a 2-min linear step to 100% acetonitrile which was maintained for 2 min, and a further 2-min linear step back to 100% [pH 3.0] water). Standards of various aromatic compounds such as pyrogallol, protocatechuic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-phenylacetic acid, 3,4-dihydroxybenzaldehyde, homovanillic acid, 3-hydroxybenzoic acid, phloroglucinol, 4-methyl catechol, 3-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid, phloroglucinol, and 2,4-dihydroxybenzoic acid were tested as possible metabolic intermediates. Chromatographic profiles of eluting compounds were obtained at appropriate dual wavelengths determined based on absorption spectral scans that ranged from 200 to 600 nm. The HPLC fractions and compounds were identified by their retention times and UV spectra in comparison to those of their corresponding standards. Fractions corresponding to the individual peaks were collected and processed separately for further analysis by MS. Controls and blanks were used prior to loading the samples. Typical HPLC profiles were established based on a minimum of four runs from three independent experiments.

ESI-MS analysis. Fractions resolved by RP-HPLC were freeze-dried, concentrated, and resuspended in 50 μ l of HPLC-grade methanol before injection into

TABLE 2. Growth characteristics of wild-type *P. putida* PML2 strain and *Flav* mutant strains in different carbon sources at 60 h postinoculation^a

Medium and/or carbon source(s)	Growth on strain:			
	WT	<i>Flav</i> -1	<i>Flav</i> -2	<i>Flav</i> -3
Tryptic soy broth	++++	++++	++++	++++
SM + 0.5% glycerol	+++	+++	+++	+++
SM + 10 mM quercetin	+++	—	—	—
SM + 10 mM naringenin	+	—	—	—
SM + 10 mM protocatechuate	+++	+++	++	++
SM + 10 mM <i>p</i> -hydroxybenzaldehyde	+++	+++	+++	+++
SM + 10 mM <i>p</i> -hydroxybenzoic acid	+++	+++	+++	+++
SM + 10 mM 3,4-dihydroxycinnamic acid	+	+	+	+
SM + 10 mM phloroglucinol	+	+	+	+

^a WT, wild-type *P. putida* PML2 strain; *Flav*-1 to -3, flavonoid utilization-defective mutant strains 1 to 3. Growth: +++++, $A_{600} > 0.6$; +++, $A_{600} \leq 0.6$ but > 0.25 ; ++, $A_{600} \leq 0.25$ but ≥ 0.1 ; +, $A_{600} < 0.1$ but > 0 ; —, no growth.

the electrospray ionization (ESI) mass spectrometer. A PE Sciex API300 LC/MS (PE Sciex, Toronto, Ontario, Canada) single-quadrupole instrument equipped with an electron spray ion source was used for analysis of masses of the breakdown products. All MS analyses were carried out in the positive ionization mode. The ring voltage was 150 V, and the mobile phase was a gradient of 1:1 acetonitrile-water containing 1 mM ammonium acetate. An Apple Macintosh system 8.1 with Mass Chrom (PE Sciex) software was used for data acquisition and processing. Spectra were analyzed and interpreted using BioMultiview 1.3 (PE Sciex). Sample analysis was performed in triplicates.

NMR analysis. NMR measurements were carried out with a 300-MHz Bruker ACF300 (Bruker Analytik GmbH, Rheinstetten, Germany) equipped with an autotunable 5-mm Quad-Nuclei QNP probe for the detection of ^1H , ^{13}C , ^{19}F , and ^{31}P , with a variable temperature facility. A sample changer was used to perform one-dimensional NMR scans for 1-h time periods. ^1H and ^{13}C NMR spectra were recorded at 298 K. All of the samples were dissolved in deuterated DMSO ($\text{DMSO}-d_6$). The chemical shifts were expressed as δ values (parts per million) by using the solvent as an internal reference ($\text{DMSO}-d_6$: $\delta_{\text{H}} = 16.22$, $\delta_{\text{C}} = 77.40$). The spectral width was set at 200 ppm. Deuterated water was used to blank water peaks.

RESULTS

Isolation of mini-Tn5gfp mutant strains. A flavonoid-utilizing PGPR strain, *P. putida* PML2 (Table 1), was identified by its ability to grow on quercetin and naringenin as a single carbon source supplement to SM (Table 2). The wild-type strain alone was additionally tested and found to be capable of growth in other flavonoids, such as daidzein, an isoflavone; apigenin, a flavone; and hesperetin and naringin, flavanones (data not shown). This strain was mutagenized by using mini-Tn5gfp. A pool of 134,000 transposants was derived from the prototrophic strain *P. putida* PML2. Transposants were obtained with a frequency of 10^{-4} compared to spontaneous mutation frequencies of $<9 \times 10^{-7}$ for *P. putida* PML2 and $<7 \times 10^{-7}$ for *E. coli* SM10 (λpir) strain. Three independently derived flavonoid-auxotrophic strains that were identified based on their inability to grow on quercetin- and naringenin-containing medium were designated *Flav*-1 to -3 and were chosen for further studies (Table 2).

Growth experiments and depletion studies. Table 2 shows the growth characteristics of *P. putida* PML2 and its three *Flav* mutant strains on a rich medium and on SM supplemented with eight substrates as carbon sources; one is a standard

carbon source, two are flavonoids, one is a standard nonflavonoid carbon source, and five are commonly reported metabolic intermediates of aromatic pathways. All of the strains described above showed comparable growth in tryptic soy broth and SM containing glycerol. Growth of the *P. putida* PML2 wild-type strain was better in quercetin than in naringenin; the maximum A_{600} values of the culture remained below 0.1 in the latter. In the common metabolic intermediate protocatechuate, the growth of *P. putida* PML2 was comparable to that in quercetin. However, in contrast to the lack of growth of the *Flav* strains in quercetin, their growth in protocatechuate was moderately high (Table 2). The wild-type PML2 strain and all three *Flav* strains utilized three of the four single-ring aromatic substrates efficiently. The cultures reached A_{600} values of >0.6 and were white. The above observations indicated specific blocks in the flavonoid metabolic pathway that affected flavonoid utilization drastically, affected protocatechuate utilization moderately, and remained unaffected in the utilization of two single-ring substrates used in this study. In order to classify the three *Flav* strains further, their rate of growth and flavonoid degradation were studied.

Growth studies of wild-type strain PML2 and its three *Flav* mutants in SM containing 10 mM quercetin (SMQ) (Fig. 3A) and in SMG (Fig. 3D) were monitored over a 60-h time period. In SMG, these three mutant strains had growth comparable to that of the wild-type strain PML2, although its growth in naringenin was moderately low (Fig. 3C). However, the three *Flav* strains still showed distinctly slow or no growth in this substrate.

In order to study the rate of quercetin utilization by wild-type PML2, depletion studies were conducted for the same time period. We used a subsaturation concentration of quercetin (0.1 mM) as the substrate that was lower than the 10 mM used in the growth studies (Fig. 3A and Table 2) to allow monitoring of significant changes in depletion levels. Quercetin levels were quantitated by ESI-MS so as to achieve high-precision measurements. The rate of depletion of 16.33 mmol/h was achieved concomitantly with the exponential growth phase of strain PML2 between 24 and 48 h postinoculation. At this rate, 0.1 mM quercetin was depleted to near completion (98%) within 60 h of incubation (Fig. 3B). Also, in comparison to the wild-type PML2 strain, as expected, *Flav*-1, -2, and -3 strains were not able to grow on naringenin (Fig. 3C) and were chosen for degradation studies and molecular characterization.

Confirmation of transposon insertions. The presence of mini-Tn5gfp was studied in the three *Flav* strains by using DNA amplification and hybridization-based assays. DNA amplifications with transposon-specific primers (Gm^r and *gfp* genes, respectively) were used to confirm mini-Tn5gfp integration into the genome. The expected 0.7-kb DNA fragments from either gene were observed in *Flav*-1, -2, and -3 strains but not in the wild-type genomic DNA (data not shown). DNA gel blot analysis was used to determine both the number of mini-Tn5gfp insertions and their positions in the genome. Hybridization of a Southern blot of three *Flav* strains genomic DNA digested with *Kpn*I or *Bgl*II that cut once in the transposon showed only single bands of different sizes. The corresponding lanes for wild-type strain PML2 did not show any signal, as expected (data not shown). This indicated that mini-Tn5gfp

FIG. 3. Growth and depletion studies. (A) Growth of wild type (○) and mutant strains *Flav-1* (△), *Flav-2* (■), and *Flav-3* (●) in SMQ. The "A600" in the y axis indicates absorbance values recorded at 600 nm. (B) Degradation of 0.1 mM flavonoid by wild-type *P. putida* PML2 in the cell soluble fractions of SMQ supernatant (▲). The growth curve of the wild-type strain PML2 (○) and the flavonoid mutant strains *Flav-1* (△), *Flav-2* (■), and *Flav-3* (●) are shown. The primary y axis shows the absorbance values recorded at 600 nm, indicated as "A600." The percent depletion refers to the decrease in flavonoid concentrations over a period of time and is shown in the secondary y axis. (C) Growth of wild type (○) and mutant strains *Flav-1* (△), *Flav-2* (■), and *Flav-3* (●) in SM supplemented with 10 mM naringenin. The "A600" in the y axis indicates absorbance values recorded at 600 nm. (D) Growth of wild type (○) and mutants *Flav-1* (△), *Flav-2* (■), and *Flav-3* (●) in SM supplemented with 0.5% glycerol as the carbon source. The "A600" in the y axis indicates absorbance values recorded at 600 nm.

had been inserted as a single copy in random locations in the three *Flav* strains.

RP-HPLC analysis of flavonoid degradation products. In order to understand quercetin catabolism, flavonoid-utilization-defective mutants were used in comparative metabolic profiling. A maximum of seven peaks could be resolved in the case of cell soluble fractions, followed by two peaks from supernatant samples, whereas cell debris had no detectable metabolites. The two peaks from the supernatant belonged to quercetin and naringenin based on MS results and comparison with results for commercially available standards (data not shown). This profile remained the same throughout the 7 days of the study period. Methanolic extracts of cell soluble fractions from the wild type and *Flav-1* strain were compared at 0, 2, and 7 days postinoculation since these were the most informative based on the number of peaks resolved. In comparison to the changing profile of metabolites from the wild-type samples (Fig. 4A to C), those from the mutant strain remained mostly unchanged over a comparable period of time (Fig. 4D to F). The fractions (belonging to the ancillary peaks) were also collected and analyzed by MS and found to have low ionization. The compounds were therefore not detected and thus determined to be insignificant. In the case of wild-type samples, six unique peaks were detected at 2 days (Fig. 4B) postinoculation compared to that of the day zero control (Fig. 4A). Most of these peaks were undetectable in samples collected at day 7. We reasoned that metabolites corresponding to

these six peaks had accumulated as a result of flavonoid degradation by *P. putida* PML2 since quercetin degradation occurs rapidly within 60 h postinoculation (Fig. 3B). Metabolites corresponding to the six peaks from day 2 samples of wild-type culture were analyzed by MS (described below). Based on the tentative identities of the six major metabolites, four commercially available pure standards were used to compare their HPLC profiles with those of the samples from cell soluble fractions. These four metabolites were naringenin (retention time, 13 min), 3,4-dihydroxy cinnamic acid (retention time, 12 min), phloroglucinol (retention time, 9 min), and protocatechuate (retention time, 7.5 min). Peaks assigned to these metabolites are based on both comparison with pure standards and MS. The major HPLC peak, excluding that of the substrate, is of naringenin, which was also detected in the whole-cell supernatant. The fact that six peaks identified from the day 2 wild-type cultures were absent in the samples from the mutant strain showed that these compounds were not a result of spontaneous oxidation events.

ESI-MS analysis of HPLC fractions. Metabolites corresponding to the six peaks (Fig. 4B) were collected as HPLC fractions, freeze-dried, and concentrated before subjecting them to ESI-MS analysis. The mass spectra of the six metabolic intermediates showed their m/z values to be 273.2, 181.5, 155.2, 139.2, 139.1, and 127.2. Compounds in peaks 1 and 4 (Fig. 4B) corresponding to m/z values 139.2 and 139.1 were not studied further due to their limiting amounts as obtained by HPLC.

FIG. 4. RP-HPLC elution profiles of phenolic fractions from the cell soluble fractions of wild-type and mutant cultures at different time points of growth. "A286" in the y axis indicates the wavelength (286 nm) used for detection. (A) Wild-type culture samples on day 0 (W-0d). (B) Wild-type culture sample at day 2 (W-2d). (C) Wild-type culture sample on day 7 (W-7d). (D) *Flav* mutant culture sample on day 0 (M-0d). (E) *Flav* mutant culture sample at day 2 (M-2d). (F) *Flav* mutant culture sample on day 7 (M-7d).

Molecules were assigned tentative names corresponding to m/z values based on comparison with existing compounds in the public database (<http://www.chemfinder.com>). Comparison of mass spectra and HPLC profiles of pure standards for four of these six metabolites allowed us to assign probable structures. The structures of these four key compounds were further confirmed by NMR spectroscopy.

NMR analysis and identification of metabolites. Four individual fractions identified by ESI-MS were subjected to one-dimensional NMR analysis in a DMSO- d_6 solvent system. ^1H - and ^{13}C -NMR measurements were carried out, and the NMR spectra were recorded at 298 K. Chemical shifts were expressed as δ values by using the solvent as an internal reference (DMSO- d_6 ; $\delta_{\text{H}} = 16.22$, $\delta_{\text{C}} = 77.40$). Based on the NMR data and the m/z values, the structures of the compounds were confirmed. They were identified as naringenin (4',5,7-trihydroxy flavanone) with δ_{H} values of 5.45, 5.80, 5.80, 6.70, 6.80, 7.29, and 7.30 ppm and δ_{C} values of 78.3, 94.8, 95.6, 98.0, 98.3, 105.0, 114.9, 115.0, 128.2, 128.7, 157.6, 162.8, 163.3, 166.5, and 196.2 ppm; caffeic acid (3,4-dihydroxycinnamic acid) with δ_{H} values of 6.2, 6.7, 6.9, 7.0, and 7.45 ppm and δ_{C} values of 114.0, 115.0, 115.6, 121.0, 125.6, 144.4, 145.0, 147.9, and 167.7 ppm; 2,4,6-trihydroxy benzene (phloroglucinol) with δ_{H} values of 5.65, 5.65, and 5.65 ppm and δ_{C} values of 93.9, 93.9, and 93.9 ppm; and 3,4-dihydroxy benzoic acid (protocatechuic acid) with δ_{H} values of 6.8, 7.2, and 7.3 ppm and δ_{C} values of 115.0, 116.0, 120.0, 120.0, 144.0, 150.0, and 166.0 ppm.

FIG. 5. Growth of wild type (○) and mutant strains *Flav*-1 (△), *Flav*-2 (■), and *Flav*-3 (●) in SM supplemented with different metabolic intermediates (10 mM). The "A600" in the y axis indicates absorbance values recorded at 600 nm. (A-CIN) Growth in 3,4-dihydroxycinnamic acid. Slower growth is seen. (B-PHL) Growth in phloroglucinol. Slower growth is seen. (C-PTC) Growth in protocatechuic acid. A relatively higher growth of wild-type and mutant strains than on the other two substrates can be seen.

Growth in metabolic intermediates. We studied the growth of wild-type PML2 and *Flav*-1, -2, and -3 strains in three aromatic intermediates determined by the above-described studies: 3,4-dihydroxycinnamic acid, phloroglucinol, and protocatechuic acid. The wild-type strain and mutant strains had comparable levels of growth in SM supplemented with 3,4-dihydroxycinnamic acid, phloroglucinol, and protocatechuic acid (Fig. 5). Both the wild-type PML2 and *Flav* strains reached a maximum A_{600} value of 0.06 in SM supplemented with dihydroxycinnamic acid (Fig. 5A-CIN) and phloroglucinol (Fig. 5B-PHL), while the A_{600} values attained by both wild-type and

mutant strains in protocatechuate were slightly greater than 0.25 (Fig. 5C-PTC), indicating that protocatechuate could be the physiologically preferred substrate, while 3,4-dihydroxycinnamic acid and phloroglucinol are not. Taken together with data shown in Fig. 3, it is seen that among the four intermediates detected in this study, *Flav*-1, -2, and -3 mutant strains were unable to utilize three of them, placing their respective blocks in the flavonoid degradation pathway before protocatechuate formation. Studies to characterize the flavonoid catabolic genes in *P. putida* PML2 are in progress.

DISCUSSION

We have described a strain that can utilize a wide range of flavonoids, including flavonols, flavanones, flavones, and isoflavones. One of the most readily used was the archetypal flavonoid compound quercetin. Quercetin, apart from its universal presence in vascular plants and root exudates (37), is also described as a major component of human food. Thus, it is not surprising that bacterial species would have developed mechanisms for utilizing it. Several microbes are already known to participate in the breakdown of aromatic compounds and flavonoids. These have been reported from two ecological niches. Those from the soil include *Rhizobia* and *Agrobacterium tumefaciens* (39), a thermophilic *Bacillus* sp. (8), a soil pseudomonad (1, 22, 23), a *Rhodococcus* strain (52), and a fungal strain of *Aspergillus niger* (41). Other microbes from the anoxic environment of the intestine include *Clostridium* strains (55), *Eubacterium* spp. (27), and a *Butyrivibrio* sp. (26). Flavonoid degradation pathways have been well studied in the intestinal flora. As in most other cases involving degradation of aromatics, studies on uptake and detection of intermediates and accumulation of end products led to the elucidation of the pathway (5, 9). In contrast to the intestinal microflora, soil pseudomonads survive better in aerobic conditions. Under such conditions, flavonoids are highly reactive. The strategy used in the present study took into consideration the reactive nature of flavonoids and specifically of quercetin due to the presence of free phenolic -OH groups. The -OH moieties enable selective glycosylation of flavonoids to generate further complexities. Additionally, the -OH groups render flavonoids capable of spontaneous oxidation under prolonged aerobic conditions, which becomes more rapid in alkaline environments. In order to eliminate the effects of products arising spontaneously in our experiments, we included comparisons of metabolic profiles of phenolic compounds from the wild-type and mutant strains that were unable to utilize flavonoids.

A pathway is described here for the degradation of quercetin by *P. putida*. The results based on the identification of metabolic intermediates and accumulation of end products validates this pathway (Fig. 6). The initial step in the degradation of quercetin in most of the aerobic pathways involves oxidation and/or reduction reactions of the parent molecule that leads to the formation of protocatechuate or catechol. It is interesting to note the initial reaction of 3,3'-didehydroxylation in the above pathway. This, as an initial step, is unusual for aerobic reactions and has been reported from rare cases. Previously, 7 α -dehydroxylation of cholic acid by *Eubacterium* and *Clostridium* spp. (4, 14) and 21-dehydroxylation of deoxycorticosterone by *Clostridium paraputrificum* have been reported in anaerobic

FIG. 6. Quercetin degradation pathway in *P. putida* strain PML2. (I) Quercetin (3,3',4',5,7-pentahydroxy flavone). (II) Naringenin (4',5,7-trihydroxy flavanone). (III) Unstable intermediate (transient product; detected by mass spectrometry). (IVa) Phloroglucinol. (IVb) 3,4-Dihydroxycinnamic acid. (V) 3,4-Dihydroxy styrene. (VI) Protocatechuic aldehyde. (VII) Protocatechuic acid. The identity of all compounds except compounds III, V, and VI was confirmed by NMR spectroscopy. All compounds were detected in the wild-type strain PML2 but not in the *Flav*-1, -2, and -3 strains and are stably formed except for compound III. Hydrolysis and cleavage of ether and keto bonds and the presence of an unstable intermediate (compound III) have been inferred based on the structures of compounds II and IV.

environments (6). Our results clearly show that quercetin is dehydroxylated to naringenin before being converted further to smaller compounds. The *P. putida* pathway described here, therefore, shows a general reversal compared to the flavonoid biosynthetic pathway in plants, where quercetin is formed by hydroxylation of naringenin by the enzyme flavanone-3-hydroxylase. In plants, the pentahydroxy molecule quercetin undergoes further reactions to produce more stable compounds

such as tannins, cyanidin, and anthocyanins. In *P. putida* PML2, quercetin degradation follows the same convergence to protocatechuate-mediated breakdown as seen in the upper pathways for the degradation of 4-coumerate, 4-chlorobenzoate, cyclohexanecarboxylate, shikimate, 4-hydroxybenzoate, quinate, benzoate, vanillate, ferulate, coniferyl alcohol, *p*-cresol, and several others (reviewed in reference 21).

The ether bonding of the flavone moiety could be the most susceptible part for ring cleavage, as expected. Hydrolytic cleavage of the ether bond at the 1,2-position of naringenin forms an unstable intermediate. A study of anaerobic transformation of quercetin to produce naringenin and other metabolites suggested the involvement of CO₂ for ring cleavage (43). In our case, the most likely mechanism seems to be the hydrolytic cleavage of the keto bond of this unstable metabolite, yielding two aromatic compounds through the carboxyl functional group. Carboxylation of the aromatic ring, followed by an H₂-mediated reductive destabilization of the ring system, has been previously described in the anaerobic degradation of phenol and other aromatics (7, 15, 19, 47). Also, anaerobic transformation of quercetin by *Eubacterium rumulus* involves similar reduction and dehydroxylations to yield eriodictyol via dehydroquercetin (43). Most studies of the anaerobic transformation of flavonoids report the formation of phloroglucinol (10, 43, 44), while others do not (17, 26). Also, several studies show that a ruminal isolate *Eubacterium oxidoreducens* converted phloroglucinol further to acetate and butyrate via dihydrophloroglucinol (27, 28, 29). However, in the metabolic profiles shown here, phloroglucinol is detected even after long incubation periods, indicating that phloroglucinol is the conserved A-ring product of quercetin catabolism in aerobic conditions and does not undergo rapid degradation. Yet another recent study on ESI-MS fragmentation of quercetin reported similar breakdown patterns, suggesting phloroglucinol formation (24). However, the slow degradation of cinnamic acid and phloroglucinol by the wild-type strain PML2 suggests that they may not be the preferred physiological substrates. Breakdown of cinnamic acid and phloroglucinol in the pathway could perhaps be the rate-limiting steps in the degradation of quercetin. We have not studied further the degradation of phloroglucinol, although some observations on its conversion to acetate and butyrate are documented (28). Specific labeling of molecules at the junction of cleavage might provide additional insight into the ring cleavage reactions and intermediates.

The B-ring intermediate 3,4-dihydroxycinnamic acid undergoes decarboxylation in aerobic conditions to yield 3,4-dihydroxystyrene and CO₂ according to the pathway described here. Styrene has been previously shown to occur naturally in the environment as a result of decarboxylation of cinnamic acid and formaldehyde (45). Two major routes of aerobic styrene degradation are known to exist in several microbes (1, 20, 52) but proceed via an initial side chain oxidation leading to the formation of phenylacetic acid in the genus *Pseudomonas* (33). Another recent study based on ESI-MS of flavonoid molecules suggested the formation of such stable ortho-dihydroxylated compounds due to the loss of -CO and -H from similar metabolites to form a nonaromatic acid (25). In our case, oxidation of the ortho-dihydroxylated styrene resulted in formation of protocatechuic aldehyde and formic acid, which upon oxidation results in protocatechuate. However, an A-ring

cleavage mechanism of quercetin in *P. putida*, yielding protocatechuate and oxaloacetate, has previously been proposed (38, 39). Justesen and Arrigoni (24), based on ESI-MS fragmentation patterns of quercetin, suggested the formation of 3,4-dihydroxybenzaldehyde and protocatechuate. Protocatechuate, the conserved B-ring degradation product, has been previously shown to serve as a sole carbon source for some pseudomonads and rhizobia after conversion to succinyl coenzyme A and acetyl coenzyme A via 3-oxoadipate and enters the TCA cycle (35). Protocatechuate has also been implicated as a central intermediate in the degradation of toluene, DDT, biphenyls, benzoate, 4-methoxy benzoate, 2,4-dichlorobenzoate, phenanthrene, phthalate, and vanillin and in the biosynthesis of phenylalanine. C-ring cleavage of quercetin and other *nod* gene-inducing flavonoids by *Rhizobia* sp., yielding phloroglucinol and protocatechuate as breakdown intermediates under aerobic conditions, is known to yield protocatechuate (38, 39), which enters the energy-yielding TCA cycle via a *meta*-cleavage pathway.

Understanding gained from this work will be useful in studying the degradation and modification of natural products from plants. Toward this end, genes involved in this pathway are being characterized.

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