Naringenin degradation by the endophytic diazotroph Herbaspirillum seropedicae SmR1

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Several bacteria are able to degrade flavonoids either to use them as carbon sources or as a detoxification mechanism. Degradation pathways have been proposed for several bacteria, but the genes responsible are not known. We identified in the genome of the endophyte Herbaspirillum seropedicae SmR1 an operon potentially associated with the degradation of aromatic compounds. We show that this operon is involved in naringenin degradation and that its expression is induced by naringenin and chrysin, two closely related flavonoids. Mutation of fdeA, the first gene of the operon, and fdeR, its transcriptional activator, abolished the ability of H. seropedicae to degrade naringenin.

INTRODUCTION

Flavonoids are phenolic compounds produced as secondary metabolites by the phenylpropanoid pathway of vascular plants. These compounds can act as antioxidants, free radical scavengers and chelators of divalent cations, and are also involved in protection against pathogens and predators (Williams & Grayer, 2004; Cook & Samman, 1996; Reddy et al., 2007). The basic structure of flavonoids contains 15 carbon atoms arranged in three rings: two benzene rings (A and B) connected by a pyran ring containing an oxygen atom, the C ring. The oxidation level and additions to the C ring define the different classes of flavonoids, while differences in the rings A and B are characteristic of each individual member (Reddy et al., 2007). The many classes include flavonols (quercetin and kaempferol), flavones (crysir, apigenin and luteolin), flavonones (naringenin), isoflavones (daidzein and genistein), aurones, anthocyanidins and chalcones (Andersen & Markham, 2006).

Apart from their function in plant physiology, diverse flavonoids are involved in plant–bacterial interactions. The best-known such interaction promoted by flavonoids is the legume–rhizobia symbiosis, in which root-exuded flavonoids act both as chemoattractants and as signalling molecules for symbiotic rhizobia. The rhizobial transcriptional activator protein NodD binds to specific flavonoids, and the transcriptionally active complex activates the expression of nod genes, leading to the production of Nod factors and triggering a regulatory cascade that culminates in the development of nitrogen-fixing nodules (Brennic & Winans, 2005; Dénarié et al., 1992; Mulligan & Long, 1989). Flavonoids can also stimulate endophytic colonization of rice by Serratia spp. (Balachandar et al., 2006) and colonization of Arabidopsis thaliana by Herbaspirillum seropedicae (Gough et al., 1997).

Three supplementary figures are available with the online version of this paper.
In 2002, Pillai and Swarup described a pathway for quercetin degradation in Pseudomonas putida PML2, also starting by C-ring cleavage, whereas Rao & Cooper (1994) showed quercetin degradation by A-ring cleavage in P. putida DSM3226. Chalcone, isoliquiritigenin, genistein, daidzein, fisetin and other flavonoids are converted into other molecules by microbial metabolism (Das & Rosazza, 2006). In many cases, common intermediate molecules were observed in the degradation of several flavonoids by aerobic bacteria. For example, phloroglucinol and protocatechuic acid originated from quercetin (Rao et al., 1991; Rao & Cooper, 1994) and catechin (Arunanachalam et al., 2003; Hopper & Mahadevan, 1997) after C-ring cleavage. Additionally, 3,4-dihydroxyphenylacetic acid and 3-(4-hydroxyphenyl)propionic acid, as well as phloroglucinol, were detected in the degradation of other flavonoids by anaerobic bacteria (Winter et al., 1989; Schoer et al., 2003; Braune et al., 2001; Schneider & Blaut, 2000).

The degradation pathways of several aromatic compounds have been biochemically and genetically characterized in bacteria (Pérez-Pantajo et al., 2008; Jiménez et al., 2002). In many cases, the transcriptional regulatory protein involved in the expression of these pathways belongs to the LysR transcriptional regulator family, whose members need a signal molecule (substrate or intermediary metabolite) for activation. The LysR family contains regulatory proteins involved in diverse metabolic processes, such as the activator of the genes responsible for the catabolism of catechol by CatM (in the presence of inducers) in Acinetobacter calcoaceticus (Romero-Arroyo et al., 1995), activation (in the presence of the inducer cis,cis-muconate) of benzoate catabolism genes by CatR in P. putida (Rothmel et al., 1990), activation of the operons nah and sal (in the presence of salicylate), responsible for the degradation of naphthalene and salicylate by NahR in P. putida (You et al., 1988), activation of the chlorocatechol degradation pathway by ClcR in P. putida (Coco et al., 1993), and activation of 2,4-dinitrotoluene degradation by DntR in Pseudomonas sp. strain DNT (Suen & Spain, 1993).

H. seropedicae is a diazotrophic endophytic bacterium that colonizes plants of the family Poaceae (Boldani et al., 1986), as well as plants from other groups. The complete genome of H. seropedicae has been sequenced (Pedrosa et al., 2011; NC_014323), which allowed the identification of an operon containing 10 ORFs, whose proteins contain conserved domains with similarities to domains found in proteins involved in aromatic compound metabolism (Table 1). This operon was named fde and is located upstream from a gene encoding a LysR-type transcriptional activator, which is divergently transcribed (see Fig. S1, available with the online version of this paper). Here we report that this operon is involved in the degradation of naringenin and that its expression is regulated by flavonoids.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** H. seropedicae strains were grown in NBfHPN-malate medium (Klassen et al., 1997) at 30 °C and 120 r.p.m. To construct the AMM1 strain (fdeA), a 512 bp PCR fragment containing part of the coding region was cloned into pCR2.1-TOPO (Invitrogen) and used to knock out the fdeA gene by homologous recombination into the genome of H. seropedicae SmR1. For the construction of the DR2 strain (fdeR), a Km cassette (from the pKIXX vector; Pharmacia) was inserted into the Smal site present in the coding region of the fdeR gene, and the resulting plasmid was introduced into the SmR1 wild-type strain. Kanamycin-resistant colonies, resulting from single (for the AMM1 strain) and double cross recombination (DR2 strain) were isolated, and insertions were confirmed by Southern analysis (results not shown). A fdeA::lacZ fusion was obtained by cloning an 820 bp fragment, containing the entire intergenic region and part of the LysR-like gene, into the transcriptional vector pMP220 (Spánek et al., 1987), producing pSU1.

**β-Galactosidase assays.** To test the ability of aromatic compounds to induce the expression of the newly identified operon, plasmid pSU1 was introduced into the wild-type H. seropedicae SmR1 strain, and the transconjugants were grown for 24 h in NBfHPN-malate (30 °C and 120 r.p.m.). The cells were then washed, diluted (OD600 0.2) in fresh medium and challenged with aromatic compounds (0.2 mM). After 6 h incubation under the same conditions, the β-galactosidase activities were determined (Bradford, 1976; Miller, 1972). The pSU1 plasmid was also introduced into the mutant strain AMM1 to follow the expression of the operon in the presence or absence of naringenin.

**Naringenin degradation assays.** The SmR1 (wild-type), AMM1 and DR2 strains of H. seropedicae were grown in NBfHPN-malate medium containing 0.2 mM naringenin. Samples of the culture were collected at the indicated times and centrifuged, and the supernatant was filtered (0.22 μm) before analysis by TLC, in which the samples were extracted with chloroform, air-dried, resuspended in chloroform, spotted on a silica gel plate (60 Å, fluorescent indicator 254 nm) and run with chloroform/methanol (84:16, v/v). The plates were photographed under UV light (254 nm) and pixel intensities from spots of same size areas were quantified using the Image Master 6.0 software (GE Healthcare).

To confirm that the capacity to degrade naringenin depends on de novo protein synthesis, cells of the H. seropedicae strains SmR1 and AMM1 grown overnight in NBfHPN-malate were collected and suspended (OD600 1.0) in fresh medium containing naringenin (0.2 mM), with or without tetracycline (10 μg ml−1) to inhibit protein synthesis. These cultures were incubated for 0, 1, 2, 3 or 4 h in a rotary shaker at 30 °C and the supernatants were analysed by TLC as above.

To obtain cell-free extracts, the SmR1 and AMM1 strains were incubated (initial OD600 0.2) in NBfHPN-malate medium in the presence or absence of 0.2 mM naringenin. After 6 h, cells were collected by centrifugation, washed, resuspended in potassium phosphate buffer (0.1 M, pH 7.0) and lysed by sonication. After centrifugation (20000 g, 30 min), the cleared supernatant (10 μg) was assayed for naringenin degradation in the same buffer at 30 °C with 0.2 mM naringenin for 0, 1, 2 and 14 h. As a control, the extract was incubated at 100 °C for 15 min before the assay. Reactions were extracted and analysed by TLC as above.
Table 1. Proteins encoded by the fde operon and their probable functions

Homologue results show the best and other relevant hits obtained by protein PSI-BLAST.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Peptide size</th>
<th>Functional assignment*</th>
<th>Representative homologue [strain]; accession no.; identity/similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fdeB</td>
<td>386 aa</td>
<td>COG1647: esterase/lipase [general function prediction only]; COG1506: dipeptidyl aminopeptidases/acylamoacyl peptides [amino acid transport and metabolism]; COG4012: dienelactone hydrolase and related enzymes [secondary metabolites biosynthesis, transport, and catabolism]; COG0429: predicted hydrolase of the alpha-beta-hydrolase fold [general function prediction only]</td>
<td>Hypothetical protein PP_3195 [P. putida KT2440]; NP_745393; 81/88. Hydrolases or acyltransferases (alpha/beta hydrolase superfamily) [Agrobacterium radiobacter K84]; YP_002546769; 52/66. Putative hydrolase [Pseudomonas fluorescens SBW25]; YP_002871755; 59/71</td>
</tr>
<tr>
<td>fdeC</td>
<td>308 aa</td>
<td>cd08343: C-terminal domain of type I, class II extradiol dioxygenases; catalytic domain; cd07258: C-terminal domain of 2,3-dihydroxy- p-cumate-3,4-dioxygenase; pfam00903: glyoxalase/bleomycin resistance protein/dioxygenase superfamily; TIGR02295: HpaD (3,4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase family); COG0179 (MhpD): 2-keto-4-pentenoate cyclase; TIGR02295: arylformamidase.</td>
<td>Glyoxalase/bleomycin resistance protein/dioxygenase [Burkholderia multivorans CGD2M]; ZP_03570622; 76/83. Putative dioxygenase [Burk. cenocepacia J2315]; YP_00234454; 34/49. Ring-cleavage extradiol dioxygenase [Ralstonia eutropha H16]; YP_728900; 34/47</td>
</tr>
<tr>
<td>fdeD</td>
<td>112 aa</td>
<td>cd03467: Rieske domain; cd03474: tolulene-4-monoxygenase effector protein complex; pfam03555: Rieske (2Fe–2S) domain (2Fe–2S domain); COG4638 (HcaE); phenylproprionate dioxygenase and related ring-hydroxylating dioxygenases</td>
<td>Rieske (2Fe–2S) domain-containing protein [Burk. vietnamiensis G4]; YP_00119947; 55/71</td>
</tr>
<tr>
<td>fdeE</td>
<td>376 aa</td>
<td>TIGR03219: salicylate 1-monoxygenase; pfam00700: pyridine nucleotide-disulphide oxidoreductase; PRK06183: 3-(3-hydroxyphenyl)propionate dioxygenase</td>
<td>FAD-binding monoxygenase [Azospirillum sp. B510]; YP_003452124; 68/79. Monoxygenase, FAD-binding [Novosphingobium aromaticivorans DSM12444]; YP_001165824; 39/57. YetM [Bacillus cereus H3081.97]; ZP_03266161; 30/46</td>
</tr>
<tr>
<td>fdeF</td>
<td>384 aa</td>
<td>PF07690: major facilitator superfamily</td>
<td>Major facilitator superfamily permease [Bradyrhizobium sp. BTA1]; YP_001238036; 61/76</td>
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<tr>
<td>fdeG</td>
<td>271 aa</td>
<td>COG1878: kynurenic formamidase [amino acid transport and metabolism]; pfam01499: putative cyclase; TIGR03035: aroylformamidase.</td>
<td>Cyclase family protein [Comamonas testosteroni KF-1]; ZP_03544237; 92/96. Putative cyclase [Brad. japonicum USDA 110]; NP_772699; 69/77</td>
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<td>fdeH</td>
<td>196 aa</td>
<td>pfam07888: cupin domain; this family represents the conserved barrel domain of the ‘cupin’ superfamily</td>
<td>Cupin 2, conserved barrel domain protein [Burk. multivorans CGD2M]; ZP_03570617; 79/86</td>
</tr>
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<td>fdeI</td>
<td>327 aa</td>
<td>pfam01557: fumarylcoacetate (FAA) hydrolyase family; COG0179 (MhpD): 2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway); PLN02856 and TIGR01266: fumarylcoacetase</td>
<td>Fumarylcoacetase (FAA) hydrolyase [Burk. ambifaria MC40-6]; YP_001811490; 70/78. 2-Keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway) [Agrobacterium radiobacter S4]; YP_002547322; 61/73</td>
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<tr>
<td>fdeJ</td>
<td>320 aa</td>
<td>cd05238: nucleoside-diphosphate-sugar 4-epimerase; cd08946: extended short-chain dehydrogenase reductases (SDRs) are distinct from classical SDRs; cd0527: L-threonine dehydrogenase, extended (e) SDRs; COG1087: UDP-glucose 4-epimerase [cell envelope biogenesis, outer membrane]; pfam01073: 3-beta hydroxysteroid dehydrogenase/isomerase family</td>
<td>Hypothetical protein PP_3206 [P. putida KT2440]; NP_745350; 49/65. NAD-dependent epimerase/dehydratase [Azospirillum sp. B510]; YP_003452206; 49/63</td>
</tr>
</tbody>
</table>

*COG functional categories are shown in square brackets. 
RESULTS AND DISCUSSION

Genomic organization of the fde operon

In silico analysis of the H. seropedicae SmR1 genome (Pedrosa et al., 2011; NC_014323) allowed the identification of an operon potentially involved in the degradation of aromatic compounds by the meta cleavage pathway (Table 1, Fig. S1; Pérez-Pantoja et al., 2008). Notably, no promoter-like sequences or terminators were found in the intergenic regions of the 10 genes forming the operon. Upstream from this operon a divergently transcribed gene encodes a LysR-type transcriptional regulator sharing high similarity (56%) with NodD from R. leguminosarum bv. viciae 3841, suggesting that this protein activates the expression of the divergent operon, a common feature of proteins from the LysR family (Maddocks & Oyston, 2008). Similar ORFs/operons are present in the genomes of Burkholderia (Burk.) vietnamiensis G4, Burkholderia ambifaria MC40-6, Marinomonas sp. MWYL1, Xanthobacter autotrophicus, Bradyrhizobium (Brad.) japonicum USDA110 and Bradyrhizobium sp. BTAi1, although their functions in these organisms remain unknown (Fig. S1). The organization of these genes in H. seropedicae SmR1 is identical to that of Burk. vietnamiensis G4 and Burk. ambifaria MC40-6. In Brad. japonicum USDA110 and Bradyrhizobium sp. BTAi1, a gene encoding a putative cyclase is lacking in the cluster. In all these micro-organisms, a gene encoding a LysR-type protein was found flanking the conserved gene cluster.

Transcription regulation of the fde operon

In order to test the ability of certain aromatic compounds to induce expression of the fde operon, β-galactosidase assays were performed with the wild-type SmR1 strain bearing the pSU1 plasmid. Fig. 1 shows that naringenin (4’,5,7-trihydroxyflavanone; Fig. S2) increased β-galactosidase activity 36-fold and that chrysirn (5,7-dihydroxyflavone; Fig. S2) induced it 12-fold. In contrast, the flavonoid quercetin (3,3’,4’,5,6-pentahydroxyflavone; Fig. S2) and other aromatic compounds did not alter β-galactosidase activity, suggesting a specific induction of operon expression by naringenin and chrysin.

The expression of the fde operon was further investigated by introducing the fdeA::lacZ fusion into H. seropedicae SmR1 and AMM1. The SmR1 and AMM1 strains, containing the fdeA::lacZ fusion, were grown in NFbHPN-malate in the presence or absence of naringenin (0.2 mM), and β-galactosidase activity was followed for 36 h. The fde operon was significantly induced in both strains after 6 h, although in the AMM1 strain it was three- to fivefold higher than in the wild-type (Fig. 2a, b). In addition, the highest activity in the wild-type was observed after 12 h, whereas in the mutant the activity increased continuously up to 36 h of growth. These results are consistent with a decrease in naringenin concentration in the wild-type strain (see results below), which gradually turns off the fde operon.

Naringenin catabolism in H. seropedicae

To determine the function of the operon identified in the H. seropedicae SmR1 genome, naringenin degradation assays were performed. In these experiments we observed that after 9 h of growth, the naringenin concentration in the supernatant of the wild-type SmR1 culture decreased to undetectable levels, indicating its degradation (Fig. 3a). In contrast, naringenin remained almost unaltered in the culture supernatant of the AMM1 (Fig. 3b), indicating the inability of this strain to degrade it. The results suggest that the newly identified operon is involved in the

![Fig. 1. Effect of aromatic compounds on the expression of H. seropedicae SmR1 containing a plasmid fdeA::lacZ fusion. H. seropedicae SmR1 (pSU1; fdeA::lacZ) cells were grown for 24 h, collected, washed and diluted to OD 600 0.2 in fresh medium containing different aromatic compounds (0.2 mM), and then incubated for 6 h at 30 °C. Protein concentrations and β-galactosidase activities were determined as described elsewhere (Bradford, 1976; Miller, 1972). The values shown are mean and SD of at least three experiments with three biological replicates.](image)
The catabolism of naringenin, and it was named fde, for flavonoid degradation. We transferred the fdeA gene to the AMM1 strain and followed naringenin degradation. The complemented strain had the same profile as the AMM1 strain (results not shown). Since the AMM1 strain is the result of a single cross, we believe that there is a polar effect, and consequently is not complemented by the fdeA gene alone.

To test if the LysR-type transcriptional regulator (FdeR) controls the expression of this operon, we produced a mutant strain and tested its ability to degrade naringenin. Fig. 3(c) shows that the fdeR2 (DR2) strain is also unable to degrade naringenin, supporting the proposed function of this operon. The ability to degrade naringenin was restored when the fdeR gene was introduced in trans into the DR2 strain (results not shown). This result is in agreement with the fact that the expression of the fde operon is dependent on the fdeR gene (R. Wassem and others, unpublished results). Taken together, these results confirm that the fde operon is directly involved with the degradation of naringenin.

In order to detect intermediates, *H. seropedicae* SmR1 was grown in the presence of a high naringenin concentration (2 mM), and the culture supernatant was analysed by FPLC after 24 h incubation. Several new UV-absorbing peaks were observed at 24 h, when compared with time zero (results not shown). These peaks probably correspond to intermediates of naringenin catabolism, since they were observed neither in the supernatant of the AMM1 strain nor in the wild-type culture grown in the absence of naringenin (results not shown).

To evaluate whether the capacity to degrade naringenin depends on *de novo* protein synthesis, we followed naringenin degradation in cells previously grown under non-inducing conditions, but added tetracycline as a protein synthesis inhibitor. Fig. 4 shows that in the

![Fig. 2.](http://mic.sgmjournals.org)
Fig. 3. Naringenin degradation by *H. seropedicae* SmR1, AMM1 and DR2 strains. Cells were grown in the presence of 0.2 mM naringenin and the culture supernatants were analysed by TLC (insets; silica gel 60 Å, fluorescent indicator F-254). Samples collected from the non-inoculated culture (control) at time zero contained 0.2 mM naringenin and were taken as 100% (not shown). (a) SmR1, (b) AMM1 and (c) DR2 strains.

Based on proposed pathways, there are two potential options for naringenin degradation by *H. seropedicae*. In the first one, FdeC and FdeH would be responsible for opening the C ring of naringenin, releasing two inter-mediates derived from the A and B rings for further degradation. The A ring-containing product could be phloroglucinol, which is degraded by a specific pathway, as proposed for *P. putida* PML2 (Pillai & Swarup, 2002). However, *H. seropedicae* has no genes similar to those responsible for phloroglucinol degradation in other bacteria. Hence, we believe this is the less likely of the two options. In the second potential pathway, the initial presence of tetracycline, naringenin content was unchanged in both strains, whereas in its absence there was complete exhaustion of naringenin in the wild-type strain after 4 h (Fig. 4a), but not in the mutant strain AMM1 (Fig. 4b). This result shows the dependence of naringenin consumption on de novo protein synthesis. To corroborate these results, we followed naringenin degradation in cell-free extracts obtained from the SmR1 and AMM1 strains incubated for 6 h in the presence and absence of naringenin (0.2 mM). After washing, cells were lysed by sonication and the soluble protein fraction was used to degrade naringenin (0.2 mM). The TLC analyses show that only protein extracts from pre-induced SmR1 cultures were able to degrade naringenin (Fig. 5), whereas neither of the extracts of the AMM1 strain nor non-induced SmR1 extracts degraded it. Naringenin was also not consumed in the presence of heat-inactivated extracts of both SmR1 and AMM1 strains. We also looked for naringenin degradation in three other bacterial species: *Herbaspirillum rubrisubalbicans* M1 (wild-type strain), which possesses an operon with the same organization (our unpublished results), *Azospirillum brasilense* FP2 (wild-type) and *Escherichia coli* (Top10 strain; Durfee et al., 2008), which do not bear an operon similar to *fde*. Naringenin was only consumed in the presence of the *Herbaspirillum* strains (Fig. S3), supporting the conclusion that this operon enables these strains to degrade it.

*In silico* analysis of the gene products of the *fde* operon was performed in an attempt to understand their function. The FdeC protein from *H. seropedicae* is similar to YxaG (29% identity) from *Bacillus* (*Bac.*) *subtilis*, which has quercetin 2,3-dioxygenase activity (Bowater et al., 2004). In addition, the FdeE protein exhibits similarity (29% identity) to the FAD-dependent monooxygenase YetM of *Bac. subtilis* (Hirooka et al., 2009). Furthermore, two different transcription regulators, YxaF and YetL, control the expression of several genes in *Bac. subtilis*, including *yxaG* and *yetM*, in response to the presence of flavonoids. The authors suggested that these proteins may be involved in the degradation of flavonoids (flavonols in particular) as a detoxification mechanism (Hirooka et al., 2007, 2009). Despite the low similarity between the genes of *H. seropedicae* and *Bac. subtilis*, these observations further support the involvement of the *fde* operon in flavonoid degradation.
cleavage occurs in the A ring, releasing oxaloacetate and a two-ring intermediate, similarly to *Pseudomonas* sp. or *P. putida* DSM3226 (Jeffrey et al., 1972; Rao & Cooper, 1994). In either of the two pathways, the B ring-containing intermediate could then be degraded by a pathway similar to that of *P. putida* PML2 (Pillai & Swarup, 2002), whereby FdeD and FdeE would catalyse the reaction that leads to protocatechuate, which is degraded by a conserved *pca* operon detected in the *H. seropedicae* genome. Finally, FdeA, FdeJ and FdeG could be involved in the *meta* cleavage pathway, producing intermediates for the tricarboxylic acid (TCA) cycle. This pathway is supported by RNaseq data, whereby *pca* genes are upregulated in the presence of naringenin (M. Tadra-Sfeir, unpublished results). These activities and intermediates need confirmation.

In summary, we identified the genes of *H. seropedicae* necessary for a novel flavonoid degradation pathway. These genes are organized in an operon of 10 ORFs, and the mutation of the first ORF (and in the gene encoding its transcriptional regulator) dramatically decreased the ability to degrade naringenin. Expression of the *fdeA*::*lacZ* fusion is strongly induced by naringenin and chrysin, suggesting that this gene cluster is involved in the degradation of naringenin and possibly of related flavonoids, such as chrysin.

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