

Analysis of Hydroxycinnamic Acid Degradation in *Agrobacterium fabrum* Reveals a Coenzyme A-Dependent, Beta-Oxidative Deacetylation Pathway

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The soil- and rhizosphere-inhabiting bacterium *Agrobacterium fabrum* (genomospecies G8 of the *Agrobacterium tumefaciens* species complex) is known to have species-specific genes involved in ferulic acid degradation. Here, we characterized, by genetic and analytical means, intermediates of degradation as feruloyl coenzyme A (feruloyl-CoA), 4-hydroxy-3-methoxyphenyl- β -hydroxypropionyl-CoA, 4-hydroxy-3-methoxyphenyl- β -ketopropionyl-CoA, vanillic acid, and protocatechuic acid. The genes *atu1416*, *atu1417*, and *atu1420* have been experimentally shown to be necessary for the degradation of ferulic acid. Moreover, the genes *atu1415* and *atu1421* have been experimentally demonstrated to be essential for this degradation and are proposed to encode a phenylhydroxypropionyl-CoA dehydrogenase and a 4-hydroxy-3-methoxyphenyl- β -ketopropionic acid (HMPKP)-CoA β -keto-thiolase, respectively. We thus demonstrated that the *A. fabrum* hydroxycinnamic degradation pathway is an original coenzyme A-dependent β -oxidative deacetylation that could also transform *p*-coumaric and caffeic acids. Finally, we showed that this pathway enables the metabolism of toxic compounds from plants and their use for growth, likely providing the species an ecological advantage in hydroxycinnamic-rich environments, such as plant roots or decaying plant materials.

Hydroxycinnamic acids (HCA), such as ferulic acid (FA) and *p*-coumaric acid (pCA), are common plant secondary metabolites. They are involved in the biosynthesis of plant cell walls, as they are precursors of lignin, the second most abundant terrestrial organic polymer (1). They are released in large amounts in soil during the decay of root cells (2) and are involved in plant biotic interactions (3) and thus are important environmental factors for soil- and plant-interacting bacteria. While HCA have been demonstrated to generally inhibit the growth of bacteria such as *Pectobacterium carotovorum*, *Xanthomonas pelargonii*, *Pseudomonas syringae*, *Staphylococcus aureus*, and *Escherichia coli* (4–6), they can be used as carbon sources by other soil bacteria, such as *Acinetobacter* sp. strain ADP1, *Bacillus subtilis*, *Pseudomonas fluorescens*, or *Pseudomonas putida* (7–10). In addition, while these compounds are generally strong bacterial repellents, they have been found to be chemoattractants for bacteria like rhizobia or agrobacteria (11–13). This feature likely helps the latter bacteria move toward phenolic-rich root environments (3). Consequently, the ability to sense/degrade HCA may be a favorable adaptation for root colonizers. Different FA degradation pathways have been described in numerous fungi and bacteria, namely, nonoxidative decarboxylation, side chain reduction, demethylation and oxidative coupling, and coenzyme A (CoA)-independent and coenzyme A-dependent deacetylation. The last pathway can either be nonoxidative or involve a β -oxidative step analogous to that of fatty acid catabolism (14). Coenzyme A-dependent non- β -oxidative deacetylation has been demonstrated in several *Pseudomonas* species and *Acinetobacter* sp. strain ADP1 (7, 15, 16), while a coenzyme A-dependent, β -oxidative pathway for HCA degradation has been proposed in bacteria such as *Rhodotorula rubra* (17), *Aromatoleum aromaticum* (18), and *Rhodopseudomonas palustris* (19).

We recently showed that *Agrobacterium fabrum*, a species of the *Agrobacterium tumefaciens* species complex (20–22), has several species-specific regions in the genome, including the SpG8-1b region containing 15 different genes named *atu1409* to *atu1423*. This region confers on *A. fabrum* the ability to degrade FA, since a mutant with the region deleted was no longer able to degrade the compound (23). FA degradation is specific to *A. fabrum*, and no other species of the *A. tumefaciens* complex is able to degrade it. The annotation of the *A. fabrum* genome suggests that the FA degradation pathway is similar to the CoA-dependent, non- β -oxidative deacetylation found in several *Pseudomonas* species (23). The *Pseudomonas* pathway involves several steps, including (i) the conversion of FA into feruloyl-CoA by a feruloyl-CoA synthetase (encoded by *fts*), (ii) the hydration and subsequent retro-aldol cleavage of feruloyl-CoA to produce vanillin and acetyl-CoA through an enoyl-CoA hydratase/aldolase (Ech, encoded by *ech*), (iii) the NAD⁺-dependent oxidation of vanillin into vanillic acid (VA) by a vanillin dehydrogenase (encoded by *vdh*), and (iv) the O-demethylation of VA into protocatechuic acid by an O-demethylase (encoded by *vanAB*). The protocatechuic acid is trans-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and description	Reference
<i>E. coli</i>		
DH5 α	λ^- ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> ($r_K^- m_K^-$) <i>supE44 thi-1 gyrA relA1</i>	41
<i>A. fabrum</i>		
C58	Wild type	20
C58 Δ SpG8-1b	C58 with <i>Atu1410</i> to <i>Atu1423</i> genes deleted; Neo ^r Kan ^r	23
C58 Δ Atu1415	C58 with <i>Atu1415</i> gene deleted	This study
C58 Δ Atu1416	C58 with <i>Atu1416</i> gene deleted	This study
C58 Δ Atu1420	C58 with <i>Atu1420</i> gene deleted; Neo ^r Kan ^r	This study
Plasmids		
pLARF1	Broad-host-range cosmid vector; <i>oriV_{RK2} oriT_{RK2}</i> ; Tet ^r	42
pLAFR1:: <i>Atu1415-Atu1423</i>	<i>Atu1415</i> to <i>Atu1440</i> genes cloned in pLAFR1	This study
pJQ200SK	Suicide vector; P15A <i>sacB</i> ; Gm ^r	43

formed into succinate and acetyl-CoA, which are carbon and energy sources in numerous bacteria, including *A. fabrum* C58 (11, 16, 24–29).

In this work, we characterized, by genetic and analytical means, the involvement of an *A. fabrum*-specific gene cluster (i.e., SpG8-1b) in FA degradation. Degradation intermediates and genes encoding proteins involved in the process were identified, showing that, unlike that in *Pseudomonas*, *A. fabrum* FA degradation involves a coenzyme A-dependent deacetylation analogous to β -oxidative pathways.

MATERIALS AND METHODS

Chemicals. The chemical reagents (solvent and standards) and medium components were obtained from VWR BDH Prolabo (Fontenay-sous-bois, France), Roth (Karlsruhe, Germany), and Sigma-Aldrich (St. Louis, MO, USA).

Bacterial strains, plasmids, and culture conditions. The bacteria and plasmids used in this study are listed in Table 1. *E. coli* was grown routinely with shaking (150 rpm) at 37°C in LB medium (30). When necessary, growth media were supplemented with appropriate antibiotics (10 μ g/ml tetracycline, 5 μ g/ml gentamicin, and 100 μ g/ml ampicillin). *A. fabrum* strains were grown with shaking (160 rpm) at 28°C in YPG-rich medium (yeast extract, 5 g/liter; peptone, 5 g/liter; glucose, 10 g/liter; pH 7.2) or in AT minimal medium (KH₂PO₄, 80 mM; MgSO₄·7H₂O, 0.65 mM; FeSO₄·7H₂O, 18 μ M; CaCl₂·2H₂O, 70 μ M; MnCl₂·4H₂O, 10 μ M; pH 7.2) supplemented with 10 mM succinate or HCA (2.75 mM, 4 mM, or 6 mM) as a carbon source and 10 mM ammonium sulfate as a nitrogen source (31). The media were supplemented as needed with appropriate antibiotics (2.5 μ g/ml tetracycline, 25 μ g/ml gentamicin, 25 μ g/ml neomycin, and 25 μ g/ml kanamycin).

Bacterial growth was analyzed in the presence of FA or pCA using a Microbiology Bioscreen C Reader (Labsystems, Finland) according to the manufacturer's instructions. *A. fabrum* strains grown overnight in AT medium supplemented with succinate and ammonium sulfate were inoculated at an optical density at 600 nm (OD₆₀₀) of 0.05 in 200 μ l AT medium supplemented with appropriate carbon and nitrogen sources in Bioscreen honeycomb 100-well sterile plates. The cultures were incubated in the dark for 5 days at 28°C with shaking at medium amplitude. Growth measurements (OD₆₀₀) were obtained at 20-min intervals. This experiment was performed in triplicate and repeated twice.

Construction of deletion mutants. *A. fabrum* C58 mutant strains were constructed by mutagenic PCR as described by Lassalle et al. (23). The *atu1420* and *atu1417* gene sequences were deleted and replaced by the Neo-Kan resistance gene *npIII* in the C58 Δ Atu1420 and C58 Δ Atu1417

strains, respectively. The genes *atu1415*, *atu1416*, *atu1418*, and *atu1421* were deleted from the start codon to the stop codon in the strains C58 Δ Atu1415, C58 Δ Atu1416, C58 Δ Atu1418, and C58 Δ Atu1421, respectively. The amplification primers used are listed in Table S1 in the supplemental material.

Bacterial degradation of HCA. According to the protocol described by Huang et al. (17), HCA degradation was performed in two steps: induction culture, or phase I, in which cells were inoculated at 1/10 in AT minimal medium supplemented with nitrogen, a carbon source (10 mM), and HCA (500 μ M), and degradation culture, or phase II, in which, after 24 h of incubation, cells from phase I were harvested by centrifugation at 3,250 \times g for 10 min and suspended in AT medium supplemented with nitrogen and HCA (500 μ M) without any other carbon source. After 24 h of incubation, cells were harvested by centrifugation at 3,250 \times g for 10 min and suspended in fresh medium in the presence of the same HCA. During 24 h of incubation, 300- μ l samples were collected every hour and centrifuged at 15,455 \times g. The supernatants were filtered through a 0.20- μ m regenerated cellulose membrane (Chromafil RC-20/15; Macherey-Nagel) and stored at -20°C for further analysis. Each experiment was repeated three times.

Preparation and incubation of cell extracts: assay of CoASH involvement. Cell extracts and assays were performed as previously described with some modifications (17). Cells from about 100 ml of AT medium supplemented with succinate (10 mM) and ferulic acid (500 μ M) were pelleted and resuspended in 5 ml of extraction buffer (100 mM KPO₄, pH 7, 10 mM dithiothreitol [DTT], 4 mM ATP). The cells were broken with a French press and centrifuged. Cell extracts (500 μ l) were incubated at room temperature in a reaction mixture containing 100 mM KPO₄, pH 7, 10 mM DTT, 4 mM ATP, 2.5 mM MgCl₂, 0.4 mM NADH, 0.5 mM reduced CoA (CoASH), and either 0.25 mM FA- or 4-hydroxy-3-methoxyphenyl- β -hydroxypropionic acid (HMPHP)-enriched culture supernatant. Control assays were performed under the same conditions without CoASH. The presence of FA was followed by high-performance liquid chromatography (HPLC) analyses. Each experiment was repeated three times.

HPLC-UV analyses. Culture medium samples were analyzed with an Agilent 1200 series HPLC system (Agilent Technologies) coupled to a UV-visible light (UV-vis) diode array detector (DAD) (Agilent 1200 series) and equipped with a Kromasil 100-5 C₁₈ column (5- μ m particle size; 250 mm by 4.6 mm; AkzoNobel). Analyses were done at a flow rate of 1 ml/min with an acidified (0.4% [vol/vol] formic acid in each solvent) MeOH-H₂O gradient starting at 30:70 for 3 min, going to 55:45 in 8 min, to 75:25 in 7 min, and then to 100:0 in 6 min and maintained for 5 min before returning to the starting conditions in 1 min and equilibration for 6 min. Methanolic solutions (1 mg/ml) of commercial FA, pCA, caffeic acid (CA), and VA were also analyzed as references for UV-vis spectra and retention

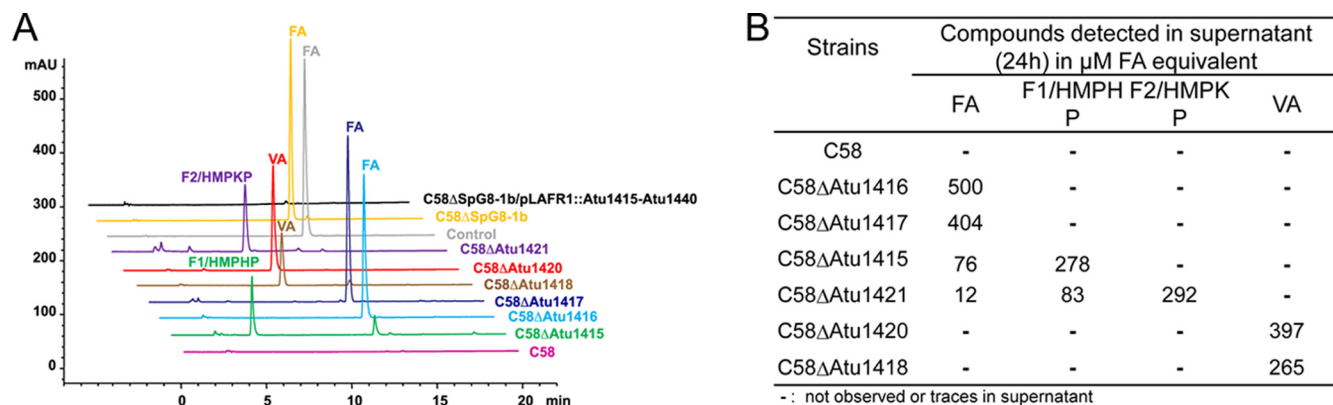


FIG 1 Involvement of the *A. fabrum* SpG8-1b region in the degradation of FA. (A) FA degradation monitored by HPLC-UV/DAD at 280 nm after 24 h. The negative control was commercial FA without inoculation. AU, arbitrary units. (B) Quantification of intermediate compounds and FA from the mutant and wild-type strains. Analyses were performed in triplicate.

times. The injection volumes were 10 μl for culture supernatants and 5 μl for standard solutions. All data acquisitions and data reworks were done at 280 nm using Agilent Chemstation software B.03.02 (Agilent Technologies).

UHPLC-UV/DAD MS QTOF analyses. Phenolic compound characterizations were performed on an ultra-high-performance liquid chromatography (UHPLC) Agilent 1290 coupled to a UV-vis DAD (Agilent 1290 Infinity series) and an Accurate-Mass Q-TOF 6530 spectrometer (Agilent Technologies). Liquid chromatography (LC) was carried out using a Poroshell 120 EC-C₁₈ column (2.7-μm particle size; 100 mm by 3 mm; Agilent Technologies) at 40°C and with injection of 3 μl of sample or 1 μl of standard solution. The mobile phase was a mixture of acetonitrile and acidified water (0.4% acetic acid) applied at a flow rate of 0.3 ml/min and the following gradient (CH₃CN-acidified H₂O [vol/vol]): starting at 7:93 for 1 min, increasing to 30:70 in 9 min, going to 100:0 in 8 min, and maintained for 1 min before returning to the starting conditions in 0.1 min and equilibrating for 3 min. The quadrupole time of flight mass spectrometry (QTOF MS) instrument was operated for MS and tandem MS (MS²) analyses under the following conditions: the ion source (Agilent Technologies Jet Stream thermal gradient focusing technology) was in positive or negative ionization mode with a 300°C gas temperature, 10-liter/min gas flow, 350°C sheath gas temperature, and 11-liter/min flow rate, with the capillary, nozzle, and fragmentor voltages at 3,000 V, 1,000 V, and 100 V, respectively. The acquisition mass range was *m/z* 50 to 2,000, and the MS² experiment was done with the collision energy set at 20 V. The LC-QTOF device was managed by the Mass Hunter Workstation Acquisition B.04.00 software, and the data reworks were done with Mass Hunter Qualitative Analysis B.04.00 software (Agilent Technologies).

Compound purifications. A C58 culture with FA (600 ml) stopped at the maximum production of the compound of interest was centrifuged, and the supernatant was extracted twice with ethyl acetate (1:1 [vol/vol]). The aqueous phase was freeze-dried and weighed. This extract was dissolved in distilled water (200 ml) and centrifuged, and the solution obtained was first separated on a Sephadex G-10 column (40 to 120 μm; 100 g; Sigma-Aldrich) with distilled water. All fractions obtained were analyzed by HPLC, and those containing the sought-after compound were pooled and freeze-dried. This compound was further purified by semi-preparative HPLC on a Nucleodur 100-5 C₁₈ column (5-μm particle size; 250 mm by 10 mm; Macherey-Nagel) with elution over a MeOH-H₂O gradient (0.4% formic acid in each solvent) from 20:80 to 100:0 (vol/vol) at a flow rate of 3 ml/min.

NMR analyses of purified compound. ¹H-¹³C gradient-selected correlation spectroscopy (gCOSY), gradient-selected heteronuclear single quantum coherence (gHSQC), and gradient-selected heteronuclear multiple bond correlation (gHMBC) nuclear magnetic resonance (NMR) spectra were recorded at 300 K with a 7 mg/ml solution in dimethyl sul-

foxide-*d*₆ (DMSO-*d*₆) using a Bruker Avance 400 spectrometer (¹H, 400.13 MHz; ¹³C, 100.61 MHz) equipped with a 5-mm BBFO+ (broad-band fluorine observation) probe with a z gradient coil. Chemical shifts are given in parts per million and referenced to tetramethylsilane as an internal standard. Data were acquired and processed with TopSpin software (Bruker, Karlsruhe, Germany).

Gene comparison and synteny analysis. Protein sequences were compared by the BLAST algorithm to the NCBI database. Proteins were considered homologs when they presented an E value of <1.53094e-136 and amino acid identities of ≥37%. Localizations of genes in genomic sequences were compared by the Agrobacteroscope platform facility (<https://www.genoscope.cns.fr/agc/microscope/home/index.php>).

RESULTS

Involvement of SpG8-1b genes in the degradation of FA. We previously showed that deletion of the SpG8-1b region (spanning from *atu1409* to *atu1423* in strain C58 [<https://www.genoscope.cns.fr/agc/microscope/mage/viewer.php?>]) prevents FA degradation (23). Here, we complemented the mutant with a deletion, C58ΔSpG8-1b, with a pLAFR1 cosmid containing entire genes from *atu1415* to the end of *atu1440* of C58 (pLAFR1::Atu1415-Atu1440) and measured their abilities to degrade FA by following the compound in culture supernatant by HPLC-UV analyses. While the C58ΔSpG8-1b mutant with a deletion did not degrade FA, the compound was completely degraded after 12 h of incubation with the complemented mutant, as well as with the wild-type strain C58 (Fig. 1A). Similar results were obtained in experiments performed with pCA and CA (data not shown). Strains of other species of the *A. tumefaciens* complex that natively do not contain the SpG8-1b region are unable to degrade FA, while their heterologous complementation with pLAFR1::Atu1415-Atu1440 conferred on them the ability to degrade FA. Consistent results were obtained with *E. coli* (see Fig. S1 in the supplemental material). These results showed that the region *atu1415* to *atu1423* is necessary and sufficient for FA degradation.

To experimentally assess the roles of individual genes, we constructed a set of C58 mutant strains lacking SpG8-1b genes. We examined their abilities to degrade FA by following the compound and its degradation intermediates in culture supernatant by HPLC-UV analyses (Fig. 1A). The deletion of *atu1416* coding sequence (C58ΔAtu1416) prevented FA degradation (Fig. 1B). We observed slight FA degradation (around 20%) in C58ΔAtu1417,

in which the *atu1417* coding sequence was entirely deleted (Fig. 1B). The mutant with *atu1420* deleted (C58Δ*Atu1420*) completely transformed FA (500 μM) into a compound identified as VA (476 μM) in less than 12 h. This strain is unable to transform VA into other products (Fig. 1B). The *atu1418* mutant (C58Δ*Atu1418*) was able to entirely degrade FA after 24 h, but this degradation was slower than that observed for the wild-type strain (data not shown), and a small amount of VA was still detected in the supernatant. Mutant strains lacking either *atu1415* or *atu1421* were able to transform FA, and they accumulated unidentified compounds named F1 and F2, respectively (Fig. 1A and B).

Characterization of compounds F1 and F2 accumulated by C58Δ*Atu1415* and C58Δ*Atu1421*, respectively. The unidentified compounds F1 and F2 were characterized by UHPLC-UV/DAD MS QTOF and NMR analyses. The F1 UV-vis spectrum indicated an aromatic phenol ring not coupled with an unsaturated bond, unlike FA (Table 2; see Fig. S2 in the supplemental material). The negative MS spectrum showed a major ion at *m/z* 211.0646 and ions at *m/z* 423.1374 and 445.1209, which corresponded to the ionic species $[M-H]^-$, $[2M-H]^-$, and $[2M + Na-2H]^-$, respectively. In positive ionization mode, this F1 compound was observed as a major ion at *m/z* 195.0614 due to loss of an $-H_2O$ during the source ionization. Thus, F1 would be a molecule with a mass of 212 Da, its accurate mass fitting with the molecular formula $C_{10}H_{12}O_5$. The positive MS² spectrum showed a product ion at *m/z* 153.0542 (theoretical mass, 153.05462, calculated for $C_8H_9O_3$; mass error, -1.44 ppm) resulting from the loss of C_2H_2O , according to the molecular formula. Another ion at *m/z* 125.0598 (theoretical mass, 125.06080, calculated for $C_7H_9O_2$; mass error, -8.02 ppm) and the base peak at *m/z* 93.0344 (theoretical mass, 93.03458, calculated for C_6H_5O ; mass error, -2.02 ppm) could correspond to the loss of the entire lateral chain with or without a methoxy moiety (Table 2; see Fig. S2 in the supplemental material). The base peak at *m/z* 93.0344 was similar to the fragment ion at *m/z* 93.0347 observed for VA $[M + H-COOH-OCH_3]^+$. Taking these results together, we propose that the F1 structure is composed of a 4-hydroxy-3-methoxyphenyl moiety and is modified on its lateral-chain $C_3H_2O_2$ compared to FA. In NMR analyses, the ¹³C chemical shifts indicated a $-COO$ at C-9 (δ_C , 172.35 ppm), and the edited HSQC spectrum showed a $-CH_2$ and a $-CH$ at C-8 and C-7, respectively. A signal at a δ_H of 2.48 ppm (2H, d, *J* = 6.48) was observed for C-8, close to the DMSO signal and coupled to the resonance at a δ_H of 4.83 ppm (1H, t, *J* = 6.48). The signal at a δ_H of 4.83 ppm indicated an electronegative substituent on C-7 resulting from a hydroxyl group, consistent with the molecular formula (see Fig. S3 in the supplemental material). Based on these chemical data, F1 was identified as an HMPHP.

Similar analyses of F2 revealed a molecule with a mass of 211 Da, its accurate mass corresponding to the molecular formula $C_{10}H_{11}O_5$ (theoretical mass, 211.06009, calculated for $C_{10}H_{11}O_5$; mass error, 2.89 ppm). According to its fragmentation pattern, F2 should be a 4-hydroxy-3-methoxyphenyl- β -ketopropionic acid (HMPKP) (Table 2; see Fig. S2 in the supplemental material). The chemical instability of this compound did not allow its purification and NMR analysis, as HMPKP is spontaneously converted into acetovanillone, as reported by Niwa and Saburi (32).

Elucidation of the FA degradation pathway of *A. fabrum*. To determine the enzymatic reaction order involved in FA catabolism, we established the kinetics of FA degradation and followed

TABLE 2 UHPLC-UV/DAD MS QTOF analysis data for FA, F1, and F2 compounds

Compound	UHPLC-UV/DAD MS QTOF analysis				Positive ion mode				Negative ion mode				
	UV-DAD data		MS		MS ²		MS		MS		MS ²		
	Retention time ^a (min)	λ_{max} (nm)	<i>m/z</i> observed	<i>m/z</i> observed	Ionic species	Precursor ion <i>m/z</i>	Observed ion <i>m/z</i>	Ionic species ^b	<i>m/z</i> observed	Ionic species	Precursor ion <i>m/z</i>	Observed ion <i>m/z</i>	Ionic species ^b
FA ^c	8.49	324/298/240	195.0620	177.0539	$[M + H]^+$	195.0620	177.0539	$[M + H_2O]^+$	193.0530	$[M-H]^-$	193.0530	178.0292	$[M-H-CO_2-CH_3]^-$
			177.0547	149.0594	$[M + H-18]^+$		149.0594	$[M + H-H_2O-CO]^+$				134.0391	$[M-H-CO_2-CH_3]^-$
				145.0280			145.0280	$[M + H-H_2O-CH_3O]^+$					
				117.0335			117.0335	$[M + H-H_2O-CO-CH_3-OH]^+$					
				89.0396			89.0396						
F1 compound (HMPHP)	4.11	278/232	195.0614	153.0542	$[M-H_2O + H]^+$	195.0614	153.0542	$[M-H_2O + H-(C_2H_2O)]^+$	211.0646	$[M-H]^-$	211.0646	151.0420	$[M-H-(C_2H_4O_2)]^-$
			153.0544	125.0598	$[M-H_2O + H-42]^+$		125.0598	$[M-H_2O + H-(C_2H_2O)]^+$				136.0186	$[M-H-(C_2H_4O_2)]^-$
				93.0344			93.0344	$[M-H_2O + H-(C_3H_2O)-OCH_3]^+$					
								$[M-H_2O + H-(C_3H_2O)-OCH_3]^+$					
									445.1209	$[2M + Na-2H]^-$			
F2 compound (HMPKP)	6.37	308/282/232	233.0425	151.0388	$[M + Na]^+$	211.0607	151.0388	$[M + H-(C_2H_4O_2)]^+$	209.0438	$[M-H]^-$	209.0438	150.0302	$[M-H-(C_2H_4O_2)]^-$
			211.0607	123.0438	$[M + H]^+$		123.0438	$[M + H-(C_2H_4O_2)]^+$				122.0353	$[M-H-(C_2H_4O_2)]^-$
			151.0388		$[M + H-60]^+$			$[M + H-(C_2H_4O_2)-CO]^+$					

^a Retention time observed on the chromatogram at 280 nm from the UHPLC-UV/DAD MS QTOF.

^b These ionic species hypotheses were proposed on the basis of the accurate mass of the product ions given their chemical formulas and also on the literature for ferulic acid.

^c Data are similar to those described by Alonso-Salces et al. (44) and Zuo et al. (45).

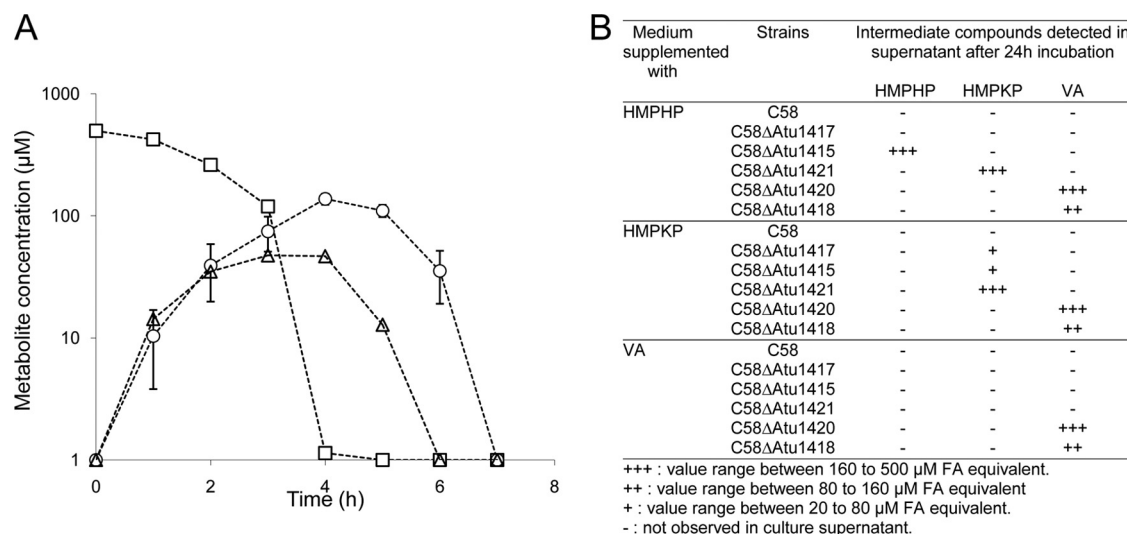


FIG 2 Intermediate degradation compounds of the *A. fabrum* HCA degradation pathway. (A) Degradation/production kinetics of FA and its intermediates. Kinetics allowed the detection of FA (□), HMPHP compounds (F1) (△), and VA (○). C58 culture supernatants were analyzed by HPLC-UV/DAD. Absorbance arbitrary units were transformed and expressed in µM equivalents by comparison with absorbance of FA for HMPHP. Bars indicate standard errors. (B) Detection of intermediates in culture supernatant analyzed from cultures incubated with the mutant strains in the presence of HMPHP, HMPKP, or VA.

the production/degradation of intermediate compounds by HPLC-UV analyses of culture supernatants. During FA degradation by the wild-type strain, HMPHP accumulated up to 47.4 µM (in µM FA equivalents) between 1 and 3.5 h after the beginning of degradation and then was completely degraded after the next 2 h (Fig. 2A). VA started to be detected a few minutes after HMPHP appearance to reach a maximum of 130 µM 5 h postinoculation and then was completely consumed during the next 2 h. VA is still observed after all HMPHP had been consumed (Fig. 2A). This observation suggested that HMPHP is a VA precursor.

We then assessed the abilities of our mutants (C58ΔAtu1415, C58ΔAtu1417, C58ΔAtu1418, C58ΔAtu1420, and C58ΔAtu1421) to degrade HMPHP, HMPKP, and VA (Fig. 2B). The mutant strains C58ΔAtu1420 and C58ΔAtu1418 accumulated VA in the presence of HMPHP or HMPKP. C58ΔAtu1420 did not transform VA at all, while VA was partly degraded by C58ΔAtu1418. Thus, VA is degraded by Atu1420 associated with Atu1418 and produced from FA, HMPHP, and/or HMPKP degradation. This confirms that VA is the last detected product of the degradation pathway. The mutant strain C58ΔAtu1421 accumulated HMPKP in the presence of FA (Fig. 1B) or HMPHP and totally degraded VA (Fig. 2B). HMPKP is thus produced upstream of VA and downstream of HMPHP and is degraded by Atu1421. Remarkably, vanillin was not detected in any of our mutants, and HMPKP has never been observed in culture supernatant except in that of the C58ΔAtu1421 mutant. The C58ΔAtu1415 strain accumulated HMPHP in the presence of FA and completely degraded HMPKP and VA. HMPHP thus has an intermediate position upstream of HMPKP and VA in the FA degradation pathway. We showed that C58ΔAtu1416 totally prevented the degradation of FA. Therefore, we proposed that Atu1416 is involved in the first step of the degradation pathway. As the C58ΔAtu1417 strain slightly degraded FA (20%) and totally degraded HMPHP, HMPKP, and VA, we proposed that Atu1417 is involved after Atu1416 and before Atu1415, Atu1421, and Atu1420.

Cell extracts were then used to test whether the ability of strain

C58 to degrade FA and HMPHP is coenzyme A dependent. HPLC-UV analyses performed after 4 h of incubation showed that in the presence of CoASH, FA was completely degraded and HMPHP and VA were detected (data not shown). In the absence of CoASH, FA was not degraded. Similar results were obtained when C58 cell extracts were incubated with HMPHP-enriched supernatant: VA was detected only in the presence of CoASH in the incubation buffer (data not shown). Hence, degradation of FA and HMPHP is coenzyme A dependent.

Taken together, all these results allow us to propose that FA was successively transformed into feruloyl-CoA, HMPHP-CoA, HMPKP-CoA, and VA by Atu1416, Atu1417, Atu1415, and Atu1421, respectively, and that Atu1420 associated with Atu1418 metabolized VA (Fig. 3).

Use of FA and pCA as carbon sources by *A. fabrum*. To measure the impact of this pathway on C58 physiology, we compared C58 and C58ΔSpG8-1b growth kinetics in the presence of FA or pCA (Fig. 4A; see Fig. S4 in the supplemental material). As the wild-type C58 strain grew slowly using these compounds as sole carbon sources (ca. 0.15 generations per day [data not shown]), we incubated the strains in minimal medium containing 10 mM succinate as an initial carbon source, in addition to FA or pCA (4 or 6 mM). Longer lag phases were observed for concentrations of ≥4 mM, suggesting FA and pCA delayed *A. fabrum* growth (Fig. 4A; see Fig. S4 in the supplemental material). The pathway encoded by SpG8-1b had no effect on reducing the growth delays induced by phenolic compounds, because the same lag phases were observed with C58 and the C58ΔSpG8-1b mutant. However, SpG8-1b allowed the use of FA or pCA as carbon and energy sources. Indeed, the maximum yields reached by C58 were higher (about 50% more) in FA- or pCA-supplemented medium than without these compounds. This was not the case with C58ΔSpG8-1b (Fig. 4A; see Fig. S4 in the supplemental material).

Growth kinetics performed with C58ΔAtu1415, C58ΔAtu1421, and C58ΔSpG8-1b mutant strains reached the same maximum yield with succinate alone or in media supplemented

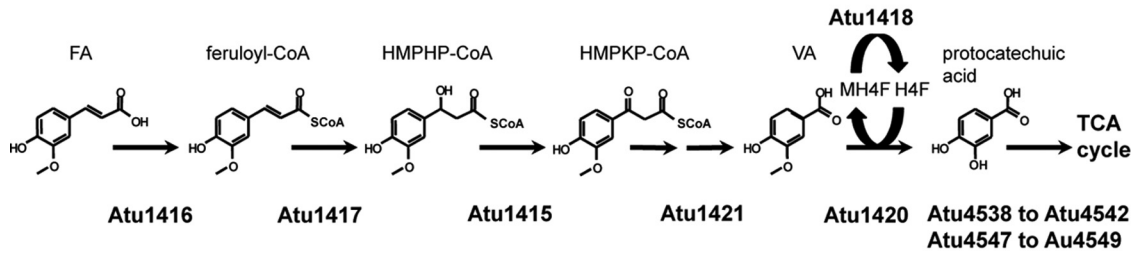
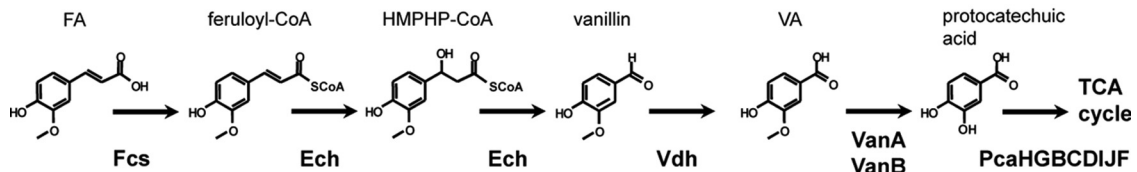
Agrobacterium fabrum HCA degradation pathway*Pseudomonas* sp. HCA degradation pathway

FIG 3 β -Oxidation CoA-dependent FA degradation pathway of *A. fabrum*. Atu1415 is the phenylhydroxypropionyl-CoA dehydrogenase, Atu1416 is the feruloyl-CoA synthase, Atu1417 is the enoyl-CoA hydratase, Atu1418 (MetF) is the methylenetetrahydrofolate reductase, Atu1420 (LigM) is the *O*-demethylase, and Atu1421 is the HMPKP-CoA β -keto-thiolase. The involvement of the *pcaHGBCD* and *pcaIJF* operons (Atu4538 to Atu4542 and Atu4547 to Atu4549) encoding proteins in C58 was previously shown by Parke (29). MH4F, methylene-tetrahydrofolate; H4F, methyl-tetrahydrofolate. For comparison, the non- β -oxidation CoA-dependent pathway described in *Pseudomonas* sp. strain HR199 (16) is presented below.

with FA or pCA. This underlines the essential functions of both enzymes for the use of FA as a carbon and energy source by *A. fabrum*. Conversely, growth kinetics performed with C58 Δ Atu1420 showed a slight increase in the presence of FA compared

to the presence of succinate alone (Fig. 4B). In the presence of pCA, the growth of C58 Δ Atu1420 was similar to that of the C58 strain (data not shown). Indeed, Atu1420 should not be involved in the degradation of this compound, since the 4-hydroxybenzo-

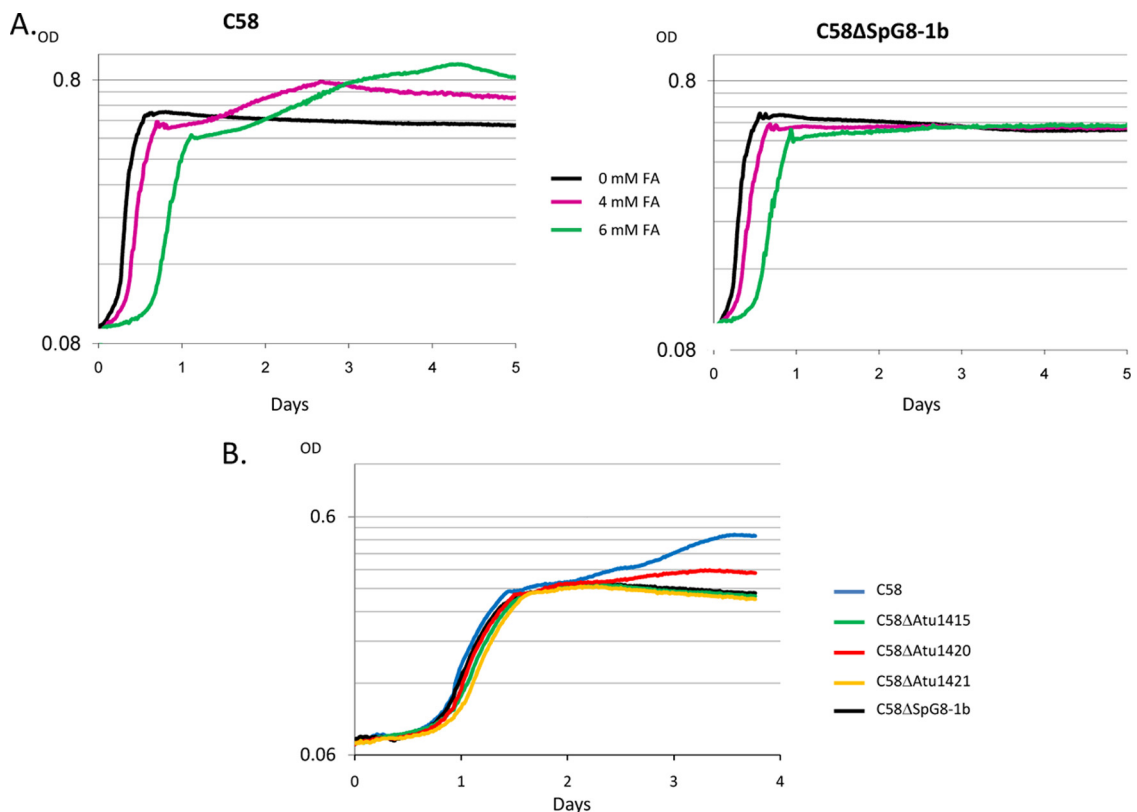


FIG 4 C58 wild-type and derivative growth curves in the presence of ferulic acid. (A) OD_{600} measured in AT medium containing succinate alone or succinate supplemented with various amounts of FA. (B) OD_{600} measured in AT medium containing succinate supplemented with 2.75 mM FA for the different strains.

ate 3-monooxygenase, PobA (Atu4544), is involved in the direct conversion of *p*-hydroxybenzoic acid from pCA into protocatechuic acid (29).

Gene and syntenic conservation among bacteria. Gene conservation analyses were performed on strains that present homologs of the first protein of the pathway (Atu1416; feruloyl-CoA synthase [Fcs]). Homologs of the other proteins of the pathway were then searched, and gene synteny was determined according to the *A. fabrum* gene organization. Thirty-three strains harbor homologs of Atu1416 (amino acid identity \geq 37%). Nine of these strains (e.g., *P. putida*, *P. fluorescens*, and *A. aromaticum*) also harbor homologs of Atu1415 and Atu1417, while 24 concomitantly harbor homologs of Atu1415, Atu1417, and Atu1421. The last strains belong to the family *Rhodobacteraceae* of *Alphaproteobacteria* and to *Betaproteobacteria*, and one strain, *Spirosoma linguale*, belongs to the phylum *Bacteroidetes*. Synteny comparisons with *A. fabrum* demonstrated that genes are colocalized two by two in the *Burkholderia* or the *Bradyrhizobium* subgroup. In six strains, the synteny is conserved for *atu1416*, *atu1415*, and *atu1417* and in one strain for *atu1416*, *atu1417*, and *atu1421*. Remarkably, 12 strains (50%) showed colocalization of the genes *atu1415*, *atu1416*, *atu1417*, and *atu1421*. In one strain (*Polaromonas naphthalenivorans*), colocalization of *atu1416* and *atu1415* and colocalization of *atu1417* and *atu1421* elsewhere in the genome were observed (see Fig. S5 in the supplemental material). The *Sagittula stellata* strain presents two *atu1416* homologs, one colocalized with *atu1415* and the other with *atu1417* and *atu1421*.

Strikingly, unlike what was observed in the *A. fabrum* genomes, no significant similarity with Atu1420 and Atu1418 could be identified in other strains harboring *atu1416* homologs, except for *Rhizobium* sp. strain NT26 and *Maritimibacter alkaliphilus* (Bio-project PRJNA53325). Moreover, it should be noted that *atu1418* and *atu1420* synteny is not conserved in the last strains. *A. fabrum* thus seems to be the only species that contains this particular gene organization with genes encoding the two steps of the pathway located close together in the same genomic cluster.

DISCUSSION

In this work, we describe a pathway that allows *A. fabrum* to metabolize FA and pCA for use as carbon and energy sources. We demonstrated that five proteins, Atu1415, Atu1416, Atu1417, Atu1420, and Atu1421, are necessary and sufficient to transform FA (and likely pCA) into protocatechuic acid, which is a known carbon source for *A. fabrum* (29).

Combining various analyses, such as phenotypic analyses, characterization of intermediate degradation compounds, and kinetic degradation analyses, we were able to construct the different steps of the degradation pathway of *A. fabrum* (Fig. 3). As annotated by Lassalle et al. (23), the first step involves Atu1416, a feruloyl-CoA synthase (Fcs) that adds coenzyme A to the FA. The second step involves Atu1417 with its enoyl-CoA hydratase (Ech) activity that converts feruloyl-CoA into HMPHP-CoA. Accumulation of feruloyl-CoA was previously shown to be toxic for *P. putida* and *Acinetobacter* sp. strain ADP1. To cope with this toxic effect and to prevent cell death for strains defective for the enoyl-CoA hydratase activity, feruloyl-CoA could be back-transformed into FA (7, 8). Accordingly, a slight decrease in FA was observed with the C58 Δ Atu1417 mutant strain that completely degraded HMPHP, HMPKP, and VA. No vanillin could be detected in our experiments and, unlike what was observed with the homologous

protein Ech from *Pseudomonas*, Atu1417 (enoyl-CoA hydratase) does not seem to convert HMPHP-CoA into vanillin. The third step involves Atu1415, which was annotated as a 3-hydroxyacyl-CoA dehydrogenase. The precise characterization of the substrate detectable as HMPHP in the supernatant using MS, MS², and NMR analyses and the coenzyme A dependence of its degradation allowed us to confirm such enzymatic activity in *A. fabrum*, and we proposed the name phenylhydroxypropionyl-CoA dehydrogenase for Atu1415. HMPHP was previously observed in the culture supernatant as a product of degradation from HMPHP-CoA in several thermophilic *Bacillus* spp. and in *Halomonas elongata*, suggesting, in these strains, thioesterase activity that released free HMPHP (33, 34). An Atu1415 homolog involved in an HCA degradation pathway in *A. aromaticum* has been recently described, but FA does not seem to be a substrate of this pathway (18). The fact that strain C58 Δ Atu1415 is unable to use FA or pCA as a carbon source argues for an essential role of the protein in the pathway. The enzyme involved in the fourth step, Atu1421, was annotated as a conserved hypothetical protein with no predicted function in the pathway (23). Strikingly, C58 Δ Atu1421 accumulated a substrate identified as HMPKP by MS and MS² and was found to metabolize VA. This prompted us to propose that Atu1421 converts HMPKP-CoA into VA, probably via the production of vanilloyl-CoA that is spontaneously converted into VA (35). Moreover, the mutant strain is unable to use FA as a carbon source, suggesting that the enzyme is essential for the pathway. Such a catabolic step has been previously suggested to be involved in the HCA degradation pathway, but to our knowledge, no experiment has been performed. We present here an experimental demonstration of an HMPKP-CoA β -keto-thiolase activity in HCA degradation. Finally, the VA accumulation in mutant C58 Δ Atu1420 confirms Atu1420 as an *O*-demethylase (LigM) that converts VA into protocatechuic acid, as predicted by Lassalle et al. (23) based upon similarities with LigM and MetF of *Sphingomonas paucimobilis* (36). This step releases free methyl, which converts methylene-tetrahydrofolate into methyl-tetrahydrofolate (37), and Atu1418 is likely the methylenetetrahydrofolate reductase involved in this regeneration (Fig. 3).

Our previous study (23) highlighted similarities between genes from the SpG8-1b region and *Pseudomonas* genes involved in FA degradation, suggesting that the degradation pathway of *A. fabrum* is a coenzyme A-dependent deacetylation. Our present work highlights major discrepancies, even though cell extract experiments showed that coenzyme A is necessary for degradation of FA and HMPHP into VA. Indeed, Atu1415 and Atu1421 were found to transform HMPHP-CoA into HMPKP-CoA and HMPKP-CoA into VA, respectively. As those steps are similar to the β -oxidation of fatty acids, the *A. fabrum* pathway is more precisely a coenzyme A-dependent β -oxidative deacetylation. The complete characterization of a coenzyme A-dependent, β -oxidative pathway done in this work confirms the occurrence of a β -oxidative step for HCA degradation suggested in *R. rubra* (17) and *R. palustris* (19) and demonstrated for hydroxylated phenylpropanoids in *A. aromaticum* (18). In addition, synteny conservation (see Fig. S5 in the supplemental material) suggests that other taxa may also degrade FA or several HCA compounds via a coenzyme A-dependent, β -oxidative pathway.

The *A. fabrum* HCA degradation pathway is not involved in pathogenicity, since C58 Δ SpG8-1b mutants are able to incite tumor formation on plants (data not shown). In studies related to

the roles of genes harbored by virulence plasmids in agrobacteria other than *A. fabrum* members, the VirH2 protein has been shown to O-demethylate FA into caffeic acid (6, 13, 38). VirH2, however, was not involved in the *A. fabrum*-specific HCA degradation pathway, since FA was not degraded by the C58ΔSpG8-1b mutant (Fig. 1) that natively harbors a Ti plasmid with a functional *virH2*. In addition, Ti plasmid-free (i.e., *virH2*-free) C58 derivatives are perfectly able to degrade FA (data not shown). For comparison, the VirH2-mediated O-demethylation of FA is very slow (starting only after 30 h of incubation) (6, 13), whereas the SpG8-1b-mediated coenzyme A-dependent β -oxidative deacetylation of FA described here was completed in less than 7 h in C58 (Fig. 2A).

Rather than a direct involvement in pathogenicity, the ecological advantage of the *A. fabrum*-specific HCA degradation pathway is likely related to cell housekeeping properties, such as detoxification or nutrition. Although, it does not have a marked detoxifying role, at least in C58 (since FA induced the same growth delay for both the wild type and mutants with deletions), it allowed the growth of *A. fabrum* using either FA or pCA (Fig. 4; see Fig. S4 in the supplemental material). Remarkably, as growth kinetics performed with C58ΔAtu1420 showed only a slight increase in the presence of FA (Fig. 4B), the first pathway steps that lead to deacetylation of FA and production of VA and acetyl-CoA thus appeared to contribute to only slight growth. Conversely, the second part of the pathway leading to protocatechuic acid, which is then processed by *pca* genes to produce intermediates of the tricarboxylic acid (TCA) cycle (29), provides the major part of the carbon and energy resulting from the complete HCA degradation. Besides *A. fabrum*, we identified homologs of *atu1416*, *atu1415*, *atu1417*, and *atu1421* in *S. stellata* E-37 (*Roseobacter* lineage). Remarkably, *S. stellata* E-37 has been shown able to degrade lignin and to use FA and pCA as primary growth substrates (39, 40). Thus, the presence of homologs of *Atu1415* (phenylhydroxypropionyl-CoA dehydrogenase) and *Atu1421* (HMPKP-CoA β -keto-thiolase) suggests their putative involvement in lignin degradation by *S. stellata* E-37 via a coenzyme A-dependent β -oxidation deacetylation of FA and/or pCA.

In conclusion, the SpG81b genomic region, which is specific to *A. fabrum*, allows members of the species to use HCA for growth through an original coenzyme A-dependent β -oxidation deacetylation. The *A. fabrum* HCA degradation pathway organization has not yet been described in any other bacteria. It seems to be optimized for the use of FA, as suspected from the remarkable grouping in a single genomic cluster of genes encoding the two pathway parts (i.e., transformation of FA to VA and then to protocatechuic acid). The growth advantage the pathway confers is specific to *A. fabrum* because the SpG81b genomic region is not present in other species of the *A. tumefaciens* complex. Consequently, the *A. fabrum* HCA degradation pathway likely confers a competitive advantage on *A. fabrum* over other members of soil *Agrobacterium* communities in HCA-rich environments, such as plant roots or decaying plant materials.

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