JOURNAL OF BACTERIOLOGY, Feb. 2011, p. 963–970 Vol. 193, No. 4
0021-9193/11/$12.00 doi:10.1128/JB.01239-10

Identification of Two Feruloyl Esterases in *Dickeya dadantii* 3937 and Induction of the Major Feruloyl Esterase and of Pectate Lyases by Ferulic Acid[▽]

Susan Hassan and Nicole Hugouvieux-Cotte-Pattat*

Université de Lyon, Université Lyon 1; INSA-Lyon; and Microbiologie Adaptation et Pathogénie, CNRS UMR5240, Domaine Scientifique de la Doua, Villeurbanne F-69622, France

Received 14 October 2010/Accepted 3 December 2010

The plant-pathogenic bacterium *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) produces a large array of plant cell wall-degrading enzymes. Using an *in situ* detection test, we showed that it produces two feruloyl esterases, FaeD and FaeT. These enzymes cleave the ester link between ferulate and the pectic or xylan chains. FaeD and FaeT belong to the carbohydrate esterase family CE10, and they are the first two feruloyl esterases to be identified in this family. Cleavage of synthetic substrates revealed strong activation of FaeD and FaeT by ferulic acid. The gene *faeT* appeared to be weakly expressed, and its product, FaeT, is a cytoplasmic protein. In contrast, the gene *faeD* is strongly induced in the presence of ferulic acid, and FaeD is an extracellular protein secreted by the Out system, responsible for pectinase secretion. The product of the adjacent gene *faeR* is involved in the positive control of *faeD* in response to ferulic acid. Moreover, ferulic acid acts in synergy with polygalacturonate to induce pectate lyases, the main virulence determinant of soft rot disease. Feruloyl esterases dissociate internal cross-links in the polysaccharide network of the plant cell wall, suppress the polysaccharide esterifications, and liberate ferulic acid, which contributes to the induction of pectate lyases. Together, these effects of feruloyl esterases could facilitate soft rot disease caused by pectinolytic bacteria.

Pectinolytic enterobacteria cause diseases in a wide range of plants, including many crops of economic importance such as vegetables and ornamentals (37). The soft rot disease produced by *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) results from the general disorganization of the plant cell wall (13). This symptom is generated by a set of bacterial enzymes able to degrade the plant cell wall polysaccharides. Pectate lyases, which depolymerize pectin, play an essential role in soft rot disease. Pectin, the major polysaccharide of the primary cell wall and the middle lamella, is necessary for the cohesion of the plant tissue. *Dickeya dadantii* produces different types of pectinases, including pectin methylesterases, pectin acetylesterases, exo- and endopectate lyases, exopolygalacturonases, and a rhamnogalacturonate lyase (13). Most of the plant cell wall-degrading enzymes are secreted by the bacteria, and it is a type II secretion system, named Out, that specifically exports most pectinases to the external medium (18). The transcription of the *pel* genes, encoding pectate lyases, is tightly regulated by environmental and metabolic stimuli (13). Pectin degradation products are the major signals allowing *Dickeya dadantii* to trigger pectate lyase induction. In addition, uncharacterized compounds present in plant extracts are able to increase the induction of pectate lyases by acting in synergy with pectin catabolic products (3, 25). Previous studies have led to the identification of several regulators controlling *pel* transcription (KdgR, PecS, PecT, Pir, CRP, Gac, etc.) that often affect other virulence factors (13, 21, 25, 28, 29, 34).

The structural complexity of the plant cell wall matrix requires the concerted action of several enzymes for its complete breakdown. In addition to pectinases, *D. dadantii* produces the cellulase CelZ, the xylanase XynA, and the endogalactanase GanA, which contribute to the degradation of plant cell wall polysaccharides (8, 18). The action of depolymerases is often limited by the presence of esterifications, which need to be removed prior to depolymerization. For example, the production of pectin methyl and acetylesterases greatly facilitates pectate lyase action in *D. dadantii* (30). Thus, esterases are considered important accessory enzymes. Feruloyl esters are a type of modification commonly found in pectins and xylans (16). Considering its large spectrum of plant cell wall-degrading enzymes, it seemed possible that *D. dadantii* might also produce feruloyl esterases (4-hydroxy-3-methoxycinnamoyl-sugar hydrolase) (EC 3.1.1.73) able to cleave this type of esterification.

Ferulic acid is the major phenolic acid esterified to carbohydrates in the plant cell wall (16). It is found mainly in arabinan and galactan chains of pectin hairy regions (5). The ferulic acid esters can form dehydrodimers, which permit covalent cross-linking of the polysaccharides that they esterify. Such diferulic acid bridges contribute to the formation of the pectic network, which is based on different types of cross-linkings (5). Enzymes responsible for cleaving the ester link between ferulate and the polysaccharidic chain have been identified in plants and associated microorganisms. Feruloyl esterases are of great interest in biotechnology for many industrial and medicinal applications (2). To date, feruloyl esterases have been characterized mainly in fungi (2). Fewer studies have been performed on bacterial feruloyl esterases,

* Corresponding author. Mailing address: Microbiologie Adaptation et Pathogénie UMR5240, batiment Lwoff, 10 rue Dubois, Domaine Scientifique de la Doua, 69622 Villeurbanne Cedex, France. Phone: 33-472-43-1553. Fax: 33-472-43-1584. E-mail: Nicole.Cotte-Pattat@insa-lyon.fr.

▽ Published ahead of print on 17 December 2010.

TABLE 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/phenotype	Reference/origin
D. dadantii strains		
3937	Wild type	Laboratory collection
A350[a]	*rafR ganB*	8, 12, 15
A1077	A350 *kdgR*::Cm	Laboratory collection
A1798	A350 *pelD*::*uidA*-Km	14
A2507	A350 *crp*::Cm	28
A3784	A350 *faeT*::*uidA*-Km	This work
A3810	*pir*::Cm	S. Reverchon
A3916	A350 *faeD*::*uidA*-Km	This work
A3994	A350 *faeR*::Cm	This work
A3959	A350 *faeD*::*uidA*-Km *faeT*::Cm	This work
A3953	*pecS*::Cm	29
A4415	*pelD*::*uidA*-Km	14
A4116	*pecT*::Cm	34
A4239	*gacA*::Cm	21
A5099	*faeD*::*uidA*-Km	This work
A5100	*faeT*::*uidA*-Km	This work
A5101	*faeR*::Cm	This work
A5123	*faeR*::Cm *faeD*::*uidA*-Km	This work
A5124	*faeR*::Cm *faeT*::*uidA*-Km	This work
A5126	*faeR*::Cm *pelD*::*uidA*-Km	This work
A5216	*faeD*::*uidA*-Km *faeT*::Cm	This work
E. coli strains		
NM522	Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5 supE thi* [F′ *proAB lacI*^{q} *lacZ*ΔM15]	Laboratory collection
BL21(DE3)	*E. coli* B, F$^-$ *dcm ompT hsdS gal* λ(DE3), T7 polymerase gene under the *lac*UV5 promoter	33
Plasmids		
pUC18	Ap^r	Laboratory collection
FE1	pUC18 derivative with a 12.3-kb insert, *faeD*$^+$	This work
FE2	pUC18 derivative with a 16.7-kb insert, *faeD*$^+$	This work
FE3	pUC18 derivative with a 17-kb insert, *faeT*$^+$	This work
pT7-5	T7 phi10, Ap^r	35
pT7-6	T7 phi10, Ap^r	35
pNA30	pT7-6 derivative with a 1.6-kb BamHI HpaI fragment, *faeD*$^+$	This work
pNA55	pT7-5 derivative with a 2.3-kb PstI SmaI fragment, *faeT*$^+$	This work

[a] The correct genotype of strain A350 (also named L2), previously indicated as *lmrT*^{c} *lacZ*, is *rafR ganB* (8, 12).

with a few enzymes described in diverse genera, such as *Bacillus* (9) or *Butyrivibrio* (7).

The potential role of feruloyl esterases in the modification of plant cell wall integrity led us to investigate whether *D. dadantii* produces such esterases. Using a functional test to detect feruloyl esterase activity, we identified two *D. dadantii* genes, named *faeD* and *faeT*, encoding such enzymes. The enzymatic activity of the proteins FaeD and FaeT was analyzed, and their cellular localization was determined. Analysis of gene expression demonstrated that ferulic acid is involved not only in the induction of *faeD* but also, in synergy with pectin catabolite products, in an increased induction of the *pel* genes encoding the major virulence factors.

MATERIALS AND METHODS

Bacterial strains and genetic techniques. The bacterial strains of *D. dadantii* or *Escherichia coli* and the plasmids used in this study are listed in Table 1. The phi-EC2 generalized transducing phage was used for transduction (27).

Media and growth conditions. Bacteria were grown in LB or in M63 medium (24). When required, the media were solidified with agar (15 g liter^{-1}). *D. dadantii* cells were usually incubated at 30°C, and *E. coli* cells were usually incubated at 37°C. Carbon sources were added at 2 g liter^{-1}. When required, antibiotics were usually added at the following concentrations: kanamycin (Km), 20 μg ml^{-1}; ampicillin (Ap), 50 μg ml^{-1}; and chloramphenicol (Cm), 20 μg ml^{-1}.

Plant extract was prepared by autoclaving 10 g of chrysanthemum leaves in 100 ml of M63 (10% extract). The extract was diluted by 10-fold in the growth medium.

Feruloyl esterase screening test. A test for screening feruloyl esterase-producing colonies was adapted from the work of Donaghy et al. (9). After 24 h of growth on M63 solid medium, colonies were covered with 6 ml of molten soft agar (4 g liter^{-1}), containing 0.05 g liter^{-1} of ethyl ferulate (ethyl-4-hydroxy-3-methoxy-cinnamate) to ensure a cloudy overlay throughout the plate. Plates were incubated at 30°C for 2 to 4 h, until a clear zone around the colonies indicated feruloyl esterase production.

Enzyme assays. Pectate lyase activity was determined by monitoring spectrophotometrically the formation of unsaturated products from polygalacturonate at 235 nm (36). Specific activity is expressed as micromoles of unsaturated products liberated per minute per milligram of bacterial dry weight (μmol min^{-1} mg^{-1}).

β-Glucuronidase activity was measured by following the degradation of *p*-nitrophenyl-β-D-glucuronide into *p*-nitrophenol at 405 nm (1). Specific activity is expressed as nanomoles of product liberated per minute per milligram of bacterial dry weight (nmol min^{-1} mg^{-1}).

Esterase activity was measured by following the degradation of *p*-nitrophenyl-acetate (PNPA) into *p*-nitrophenol at 405 nm. The appearance of the product was monitored for 20 min at 37°C. The standard assay mixture consisted of 50 mM phosphate buffer at pH 6.5, 2 mM PNPA, and the extract in a total volume of 1 ml. Specific activity is expressed as nanomoles of product liberated per minute per milligram of protein (nmol min^{-1} mg^{-1}). The K_m and V_{max} values for FaeD and FaeT were determined under the standard conditions using substrate concentrations between 0.1 and 2 mM. Different assay conditions were also tested on FaeD and FaeT activity. The optimum pH was determined using 50

mM phosphate buffer from pH 6 to 7.5. Various buffer concentrations, 50 to 400 mM, were tested. The influence of various divalent cations was assessed in the presence of Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, or Mn^{2+} at 0.1 and 1 mM concentrations relative to the corresponding chloride salt. To complex the cations, EDTA was added at a final concentration of 10 mM. The effect of ferulic acid on the esterase activity was tested at concentrations ranging from 0.1 to 0.5 mM. Under each condition, the spontaneous cleavage of the substrate was tested in parallel by omitting the extract addition.

Recombinant DNA techniques. Preparation of plasmid or chromosomal DNA, restriction digestions, ligations, DNA electrophoresis, and transformations were carried out as previously described (11). For the construction of the *D. dadantii* genomic library, the chromosomal DNA was partially digested with Sau3A, and 10- to 20-kb fragments were ligated into plasmid pUC18 previously digested with BamHI and treated with alkaline phosphatase (32).

For nucleotide sequence determination, deletions were generated with restriction nucleases, and sequence completion was performed by Eurofins MWG Operon (Grenoble, France). The nucleotide sequences of 3-kb HindIII-SalI and of 3.3-kb BglII-SmaI fragments encompassing *faeD* and *faeT*, respectively, were determined.

Genetic fusions were constructed on the cloned genes by insertion of *uidA*-Km cassettes (1) into the SacII site of *faeD* or into the Eco47III site of *faeT*. The *faeR* gene was inactivated by insertion into the internal BamHI site of the CKC15 Cm cassette (21). Plasmids were introduced into *D. dadantii* cells by electroporation. The insertions were integrated into the *D. dadantii* chromosome by marker exchange recombination after successive cultures in low phosphate medium in the presence of the appropriate antibiotic. Verification of the correct recombination of the insertions was performed by PCR.

Overproduction of the proteins FaeD and FaeT. The T7 promoter-T7 RNA polymerase system (35) was used to overproduce the esterases. The 1.6-kb BamHI-HpaI fragment overlapping *faeD* and the 2.3-kb PstI-SmaI fragment overlapping *faeT* were inserted into the pT7-6 and pT7-5 vectors, respectively. The nucleotide sequences of the resulting plasmids, pNA30 and pNA55, respectively, were verified. The plasmids pNA30 (*faeD*$^+$) and pNA55 (*faeT*$^+$) were introduced into the *E. coli* strain BL21(DE3), which contains a chromosomal copy of the T7 RNA polymerase gene under the control of the *lac*UV5 promoter (33). After *E. coli* transformation, the feruloyl esterase activity of the recombinant clones was verified by using the screening test in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG). The BL21(DE3) cells containing the plasmids were grown at 30°C in LB medium with ampicillin (200 μg ml^{-1}). When the optical density at 600 nm reached 0.4 to 0.6, the synthesis of T7 RNA polymerase was induced by the addition of IPTG at a final concentration of 2 mM, and cells were grown for an additional 2 h.

Cellular fractionation. Different cellular fractions were obtained from *E. coli* BL21(DE3)-induced cells. The bacterial cells were recovered by centrifugation for 2 min at 8,000 rpm. The pellet was suspended in 0.7 ml of 80 mM Tris-HCl buffer at pH 8.0 and 0.1% Triton X-100, and the cells were broken by sonication. Centrifugation for 2 min at 10,000 × *g* led to the recovery of the supernatant containing the soluble proteins. The pellet corresponding to the insoluble proteins and cell debris was suspended in 0.7 ml of 80 mM Tris-HCl buffer, pH 8. The periplasmic fraction was recovered by osmotic shock (6).

Analytical procedures. The protein concentrations in each fraction were determined by the Bradford method using a commercial protein assay kit (Bio-Rad) and bovine serum albumin (BSA) as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on slab gels (4% stacking gel and 12% separating gel) using the Mini-Protean II system (Bio-Rad). The proteins were revealed by staining with Coomassie blue G-250.

Pathogenicity tests. Plant infections were inoculated as previously described (12, 38). Ten chicory leaves were infected for each strain using 10^6 bacteria per inoculation site. After incubation in a dew chamber for 24 h at 30°C, the length of rotted tissue was measured to estimate the disease severity. Similarly, 10 potato tubers were infected for each strain using 5×10^6 bacteria per inoculation site. Following incubation in a dew chamber for 48 h at 30°C, the weight of rotted tissue was measured to estimate the disease severity. After infections, the plant-macerated tissue was recovered and used to perform bacterial cell numeration by dilution plating and β-glucuronidase assay (38). The specific activity was calculated as nmol of product formed per 10^9 bacteria. The wild-type strain used as a negative control showed no detectable β-glucuronidase activity. A fusion in the *pelD* gene was used as a positive reference in each experiment (14).

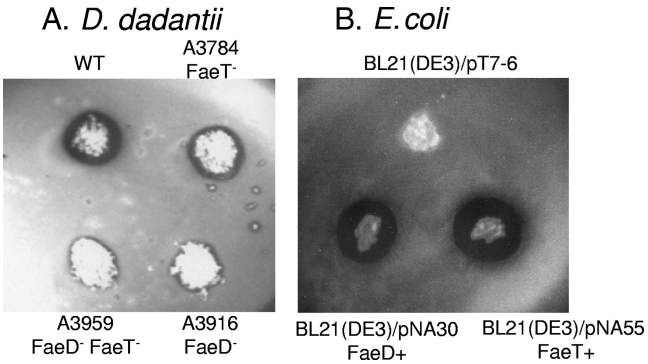

FIG. 1. *In situ* detection of feruloyl esterases. The test was performed on *D. dadantii* colonies grown on M63 medium supplemented with glycerol (A) and on *E. coli* after growth on M63 medium supplemented with glucose, Casamino Acids, and IPTG (B). The wild-type *D. dadantii* 3937 strain (WT) gave a positive response, and *E. coli* strain BL21(DE3)/pT7-6 was used as a negative control.

RESULTS AND DISCUSSION

Isolation of two *D. dadantii* genes encoding feruloyl esterases. A culture medium containing ethyl ferulate has been previously used to detect feruloyl esterase activity in *Bacillus* spp. (9). In order to detect colonies degrading ferulate esters, we adapted a similar plate assay for the enterobacterium *D. dadantii*. Since the presence of ethyl ferulate in the growth medium strongly inhibited the growth of both *D. dadantii* and *E. coli*, we overcame this inhibition by adding ethyl ferulate in an overlay poured onto the plates after 24 h of growth. The presence of this substrate resulted in an opaque appearance, and clear haloes appeared around the colonies degrading ethyl ferulate after a few hours of incubation (Fig. 1). *D. dadantii* 3937 gave a positive response to this test. In contrast, *E. coli* strains were totally negative. Consequently, this test was used to screen a *D. dadantii* genomic library introduced into *E. coli* NM522. Three clones giving a positive reaction, FE1, FE2, and FE3, were selected among about 800 transformants (10- to 20-kb inserts). FE1 and FE2 had 12.3- and 16.7-kb inserts, respectively, including a 5.5-kb common region. FE3 had a 17-kb insert distinct from those of FE1 and FE2. The esterase gene common to FE1 and FE2 was named *faeD*, and that found in FE3 was named *faeT*. These genes were localized more precisely by subcloning (Fig. 2). A second screening of the *D. dadantii* genomic library was performed on about 1,000 novel clones. Four novel positive clones were obtained, and their restriction analysis indicated that they carried the gene *faeD*. This test appeared to be specific for enzymes degrading ferulate esters, since a negative response was obtained with *E. coli* containing other known *D. dadantii* esterase genes, such as *pemA*, *pemB*, *paeX*, *paeY*, *estC*, and *estV* (data not shown).

Characterization of the *D. dadantii* genes *faeD* and *faeT*. The sequence of a 3-kb DNA fragment including *faeD* and that of a 3.3-kb fragment overlapping *faeT* were determined. The *faeD* and *faeT* sequences were identical to those reported for ID16816 and ID17388, respectively, in the 3937 genome sequence (GenBank accession no. CP002038.1). Insertion of a *uidA*-Km cassette into the SacII site interrupting *faeD* suppressed the positive response to the esterase screening test, confirming that this gene encodes the esterase activity. Simi-

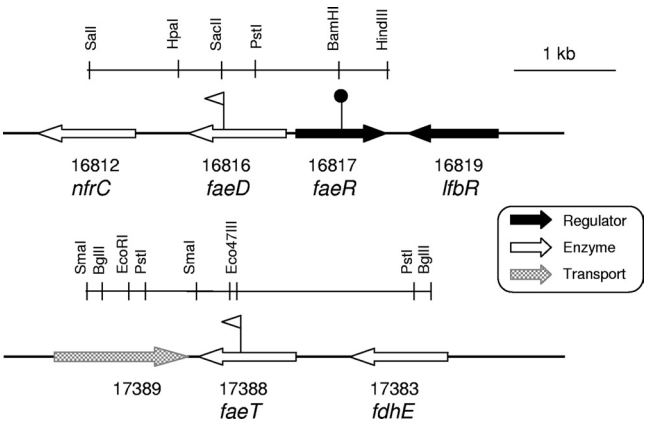

FIG. 2. Genetic organization of the *faeD* and *faeT* regions in the *D. dadantii* 3937 genome. The flags indicate the positions of the *uidA*-Km insertions, resulting in gene fusions (triangles), and the Cm insertions in the regulatory gene (circles).

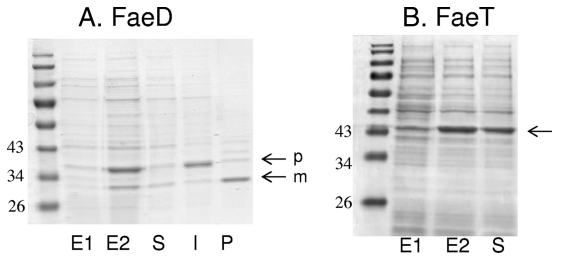

FIG. 4. Overproduction and cellular localization of the FaeD and FaeT proteins in *E. coli*. The proteins were separated by SDS-PAGE, and the gels were stained with Coomassie blue. The positions of the molecular weight standards are indicated. The arrows indicate the positions of the overproduced proteins (p, precursor form; m, mature form). (A) Overproduction of FaeD. Cell lysates of BL21(DE3)/pNA30 before induction (E1) and after induction (E2) with IPTG. Cell fractionation obtained after IPTG induction: soluble (S), insoluble (I), and periplasmic (P) fractions. (B) Overproduction of FaeT. Cell lysates of BL21(DE3)/pNA55 before (E1) and after (E2) induction with IPTG and the soluble protein fraction obtained after induction.

larly, insertion of a *uidA*-Km cassette in the Eco47III site of *faeT* confirmed the correct identification of this esterase gene.

The gene *faeD* is divergently transcribed from a gene encoding a regulator of the LysR family, which we named *faeR* (ID16817) (Fig. 2). The genes *faeD* and *faeR* are separated by 174 nucleotides (nt) and could share the same regulatory region. The gene *faeD* is followed by a sequence typical of a rho-independent termination site, and it is separated by a 581-nt intergenic space from the gene *nrfC* (ID16812) encoding an iron-sulfur protein which is part of the formate-dependent nitrite reductase complex. The gene *faeR* is adjacent to *lfbR*, encoding a previously analyzed LacI family regulator of unknown function (38). The gene *faeT* is separated by 661 nt from the upstream gene, *fdhE* (ID17383), encoding a chaperone of formate dehydrogenase, and it is separated by 99 nt from the downstream divergent gene encoding a potential transporter. Thus, considering their genetic organization (Fig. 2), both *faeD* and *faeT* probably constitute independent transcriptional units.

The genes *faeD* and *faeT* encode predicted proteins of 324 and 326 amino acids, respectively. The products of both ID16816 and ID17388 were annotated as a putative lipase/esterase (http://asap.ahabs.wisc.edu/asap/ASAP1.htm). FaeD and FaeT shared 44% identity between them and about 27% identity with the *D. dadantii* pectin acetylesterase PaeX (Fig. 3). Comparison of their sequences with those of the classified carbohydrate esterases (http://www.cazy.org/) indicated that they belong to the large family CE10. The primary sequence of these proteins includes the motif GXSXG, characteristic of the active site of serine hydrolases (4) (Fig. 3). The main differences between FaeD and FaeT are observed in their N-terminal regions. In contrast to FaeT, the N-terminal sequence of FaeD shows the characteristics of a classical signal sequence of exported proteins, with a putative cleavage site between the two alanine residues at positions 27 and 28.

The locus *faeD-faeR* is conserved in the genomes of the *Dickeya* and *Pectobacterium* species *D. dadantii* Ech586 and Ech703, *D. zeae* Ech1591, *P. carotovorum* PC1 and Wpp14, *P. wasabiae* WWPP163, and *P. atrosepticum* SCRI1043 (67 to 72% identity). The gene *faeT* is conserved in *D. dadantii* Ech586 and Ech703 and in *D. zeae* Ech1591 (79% identity). In contrast, there is no *faeT* ortholog in the *Pectobacterium* species.

Characterization of the esterases FaeD and FaeT. The *faeD* and *faeT* genes were inserted under the control of the T7 promoter in the pT7-6 and pT7-5 vectors, respectively. The resulting plasmids, pNA30 (*faeD*$^+$) and pNA55 (*faeT*$^+$), were used to overproduce the FaeD and FaeT proteins by IPTG induction in *E. coli* BL21(DE3). The feruloyl esterase plate assay showed that colonies of BL21(DE3) containing either pNA30 or pNA55 are surrounded by large haloes, while a negative response was obtained with the empty pT7-6 vector (Fig. 1). Different cellular fractions were prepared and analyzed by SDS-PAGE (Fig. 4). In the case of FaeD, two bands

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MRTLPLKMILLSAVTIITGAAQVTSAMAADNIHELTLTPRQPSVKLISDVVYAQVPMRGYANVALK----MDILQPETKSPQP------AVLFITGGGFINANKDSYI-QQRLKLAEAGYVV 110
          MANSTSVNNLMGYTKGAQVIRVKPVRQQ-IDAINDIVYSQIKSI-QKVQQLQ----MSLLVPRTTALKP------AIVYFPGGGFASAAYAKYI-DVRMALAEAGFVV  95
    MSLRRVIAGTLMMSVSGFTLADTIFPIWPQGEA-PGAATSSVQQQVVER-SKDPTLPDRAVTGIRSPEITVYTPEKPNGTALLITPGGGSYQRVVLDKEGSDLAPFFTRQGYTL 111

                                                  *                   *
ASMEYRVAPTVLF---PAPLEDVKSAIRYLRANAGKFGIDAGHVAVFGASAGGYLAAFAGTTNGNKQFDRGEHLDQSSQVQAVIDFYGLSDLTRVGEGFPDEVVQKHASPSSTEAIWVNGT 227
AAAEYRVVAEKY----PALIEDAKAAVRYLREHAAEYGIDPNRIGVLGDSAGGYVAQMTGATNGLRSFDKGRFLDKSSDVQAVVSAYGISNLLSIGEGLPENLLVVHDSPAVTEALLVNGG 212
FVMTYRMPGDGHQEGADAPLADAQRAIRTLRAHAAQWQIDPQRIGIMGFSAGGHVAASLGTR---FAQTVYPAQDEI-DHI---------------SARPDFMVLMYPVISMQENIAHAGS 208

                                  *
SVFND-GGPITRFPEKT--AAANPITYIGQHTPPFLIMHGSADTLVSPRQTEILHRAL IAHGIDSTYYVVKGAEHGGVYWLQPEIMQKVIQFLDRQLKP 324 FaeD
TAFGDFPATITSDPVKA--LNASPMGHIDGKKPPYLIMHGSADTVVSPLQSKQLYEAL VAGGNKAEYLLLEGAGHGDINWFQPVIINKIVNWFKQTLGEPITQTAPTGGNDTDGQL 326 FaeT
R-----KALIGSHPSDAQIQRYSAEKQVSAQTPPTFLVHAIDDPSVSVDNSLVMLAAL RAHQIPAEIHLFEQGKHGFGIRGTVGLPAAIWPQLLDNWLTSLPLKKNTANQPDKK 322 PaeX
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FIG. 3. Alignment of FaeD, FaeT, and PaeX. The N-terminal signal sequence and the G-S-G motif of serine hydrolases are underlined. The stars indicate the conserved residues that are putative constituents of the catalytic triad of a serine hydrolase, S, D, and H.

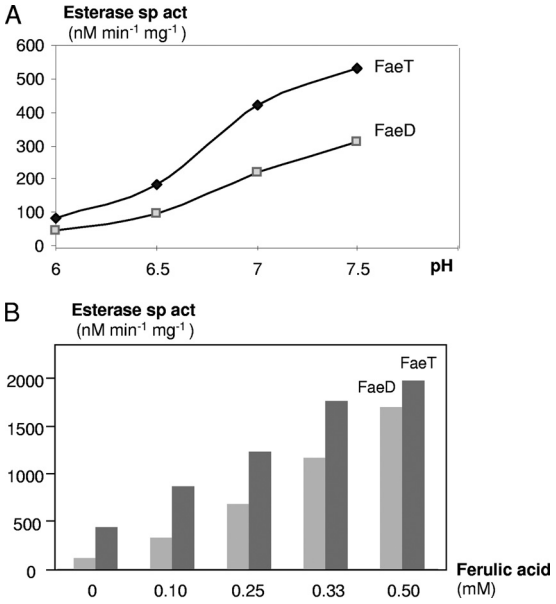

FIG. 5. Esterase activity of FaeD and FaeT. (A) Effect of pH. The esterase specific activity (sp act) was assayed with 2 mM PNPA as the substrate in 50 mM phosphate buffer at pH 6.0 to 7.5. (B) Activation by ferulic acid. The esterase assay was performed with 2 mM PNPA in 50 mM phosphate buffer at pH 6.5 in the presence of increasing concentrations of ferulic acid.

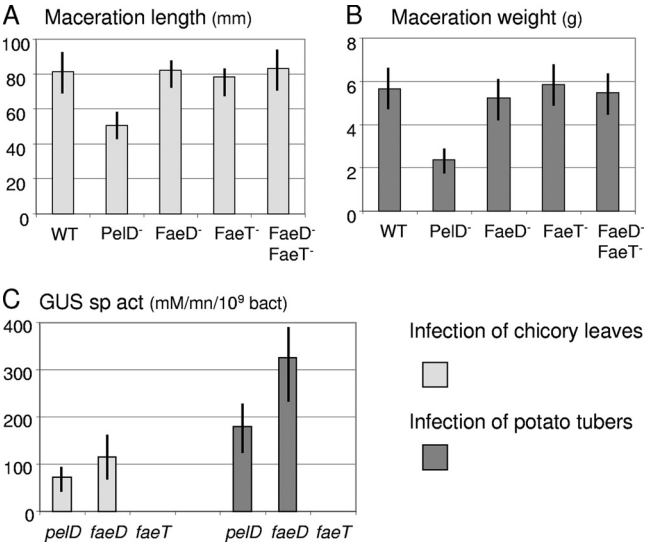

FIG. 6. Infection of chicory leaves and potato tubers with the Fae$^-$ mutants. (A) After a 24-hour infection with each mutant and the wild-type strain (WT), the length of macerated tissue was measured to estimate the disease severity. (B) Similarly, the weight of macerated tissue was measured after a 48-hour infection of potato tubers. (C) After infection, the β-glucuronidase (GUS) assay and a bacterial numeration were performed on the macerated tissue in order to estimate the expression of the *uidA* fusions in *pelD*, *faeD*, and *faeT*. The mean values and the standard deviations reported correspond to 10 infections with each strain.

of overproduced proteins were observed, with apparent sizes of 31 and 35 kDa. This result is in agreement with the data deduced from the *faeD* nucleotide sequence, predicting a precursor form of 35,045 Da and a mature form obtained after cleavage of the predicted signal sequence of 32,270 Da. The 35-kDa band was located mainly in the fraction corresponding to insoluble or membrane proteins, and the 31-kDa band was recovered mainly in the periplasmic fraction (Fig. 4). A previous analysis of the 3937 secretome demonstrated that FaeD is an extracellular protein secreted by the Out system involved in the secretion of most *D. dadantii* pectinases (18). The first step of this type II secretion is the exportation of the secreted protein through the inner membrane by the general Sec system. The conservation of this step in *E. coli* confirmed the functionality of the FaeD signal sequence.

In the case of FaeT, a single band of overproduced protein was observed. Its size of 35 kDa corresponded to that of the protein predicted from the *faeT* nucleotide sequence (34,686 Da). More than 80% of the 35-kDa protein was found in the fraction containing the soluble proteins, suggesting that FaeT is a cytoplasmic protein.

We used *p*-nitrophenyl-acetate (PNPA) as a substrate to perform esterase assays. The highest esterase activity was found in the periplasmic fraction for BL21(DE3)/pNA30 cells (FaeD$^+$) and in the soluble fraction for BL21(DE3)/pNA55 (FaeT$^+$). These fractions were retained for a biochemical analysis of FaeD and FaeT. An esterase assay at different pHs showed that the activity of FaeD and FaeT increased with the rise in pH (Fig. 5A), but the substrate became unstable at an alkaline pH. Thus, the standard assay was performed at pH 6.5 in 50 mM phosphate buffer. The initial velocities of the enzymes were determined at different PNPA concentrations under standard conditions. Apparent K_m and V_{max} values for FaeD were 1.7 mM and 625 nmol min^{-1} mg^{-1}, respectively. For FaeT, apparent K_m and V_{max} values were 2.2 mM and 1,140 nmol min^{-1} mg^{-1}, respectively.

We used various compounds to test their potential effect on the esterase activity. No difference in enzymatic activity was observed after the addition of cations (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, or Mn^{2+}) at a final concentration of 0.1 or 1 mM or of EDTA at a final concentration of 10 mM. In contrast, we observed a strong activation of both enzymes by ferulic acid (Fig. 5B). Addition of 0.5 mM ferulic acid gave a 15- and 4-fold increase of the FaeD and FaeT activities, respectively. This activation could result from a conformational change of the proteins FaeD and FaeT, giving rise to more active forms.

Phenotype and virulence of the *faeD* and *faeT* mutants. The plate assay was used to compare the feruloyl esterase activity levels of the feruloyl esterase single and double mutants (FaeD$^-$, FaeT$^-$, and FaeD$^-$ FaeT$^-$) with that of the wild-type strain (Fig. 1). When *faeT* was inactivated, the size of the esterase halo clearly decreased. When *faeD* was inactivated, only very low residual activity was detected, as observed in the *faeD faeT* double mutant. Such low residual activity could be due to nonspecific esterases. Thus, FaeD appears to be responsible for most feruloyl esterase activity in *D. dadantii*.

The extent of soft rot caused by different mutants was tested on chicory leaves and potato tubers and compared with the symptoms caused by the wild-type strain 3937 (Fig. 6). We observed no significant difference in the degree of maceration caused by the *faeD* and *faeT* mutants. Moreover, these mutations did not affect bacterial growth in the plant tissues (data not shown). Similarly, the virulence of the *faeR* mutant and the

TABLE 2. Expression of the *faeD* and *faeT* transcriptional fusions

Fusion/mutation	Potential inducer[a]	β-Glucuronidase sp act[b]
faeD::*uidA*	None	860
	Plant extract	2,672
	Ethyl ferulate	2,176
	Ferulic acid	9,691
	Polygalacturonate	911
faeD::*uidA faeR*	None	565
	Ferulic acid	557
faeT::*uidA*	None	12
	Plant extract	15
	Ferulic acid	14
	Polygalacturonate	11
faeT::*uidA faeR*	None	15
	Ferulic acid	13

[a] Strains were grown to late exponential growth phase in minimal medium containing glycerol. The potential inducers were added at the following concentrations: plant extract, 1%; ethyl ferulate, 0.1 mM; ferulic acid, 0.1 mM; polygalacturonate, 2 g liter^{-1}.

[b] The values reported (in nmol min^{-1} mg^{-1}) are the averages of results from at least 3 independent experiments, with standard deviations corresponding to less than 20%. There is no significant difference between the *faeT* data. In comparison to the noninduced value, the expression of *faeD* was significantly different ($P < 0.1$) in the presence of plant extract, ethyl ferulate, or ferulic acid.

faeD faeT double mutant on potato tubers or chicory leaves was not affected (Fig. 6 and data not shown). Transcriptional fusions with the reporter gene *uidA* were used to estimate the *in planta* expression of *faeD* and *faeT*. After infection, the expression of the *faeD* and *faeT* fusions in the macerated tissue was compared to that of the highly inducible pectate lyase gene *pelD* (14). The *faeD* fusion was transcribed in the macerated tissue at levels similar to those of *pelD* (Fig. 6C). In contrast, no expression of the *faeT* fusion could be detected. Similar data were observed for potato tuber infection. These results indicate that feruloyl esterase genes are not essential for the development of soft rot disease but that the *faeD* gene is expressed during plant infection.

Expression of the genes *faeD* and *faeT* under different conditions. Different phenolic compounds, polysaccharides, and carbon sources were added to the growth medium to reveal any potential effects on *faeD* or *faeT* transcription. The expression of the *faeD* fusion was stimulated by about 3-fold in the presence of plant extracts or ethyl ferulate and 11-fold by ferulic acid (Table 2). It was not significantly affected by the addition of acetosyringone, benzoic acid, cinnamic acid, *p*-coumaric acid, vanillic acid, arabinogalactan, arabinose, galactose, galacturonate, mannose, pectin, polygalacturonate, rhamnose, salicine, xylose, or xylan. The *faeT* fusion was weakly expressed under all the conditions, and none of the compounds tested affected its expression (Table 2). This low expression level of *faeT* was confirmed by transcriptome analysis (G. Condemine, personal communication).

The effect of ferulic acid concentrations ranging from 0.05 to 1 mM was tested on *faeD* induction (Fig. 7B). Maximal induction was reached at a concentration of 0.1 mM. Since phenolic acids are known to inhibit bacterial growth (26), concentrations ranging from 0.1 to 20 mM were tested on *D. dadantii* growth (Fig. 7A). While no inhibition at concentrations allowing *faeD* induction was observed, a clear inhibition occurred at concentrations higher than 1 mM, with both decreased growth rate and yield.

When one of the regulatory genes *kdgR*, *pecS*, *pecT*, *pir*, or *gacA* was inactivated, the expression of the *faeD* fusion remained induced in the presence of ferulic acid, with induction factors of about 12, 10, 8, 11 or 7, respectively. Thus, none of these regulators seems directly involved in the induction by ferulic acid. In the presence of the *crp* mutation, *faeD* expression decreased by about 7-fold but remained weakly induced by ferulic acid. The gene *faeR*, which is divergent from *faeD* and encodes a LysR family regulator, was inactivated. The *faeD* fusion was introduced in the *faeR*::Cm mutant. When *faeR* was inactivated, the expression of the *faeD* fusion decreased, and it was no longer inducible in the presence of ferulic acid (Table 2). The expression of the *faeT* fusion remained low; it seemed to be unaffected by *faeR* inactivation (Table 2). Thus, FaeR is an activator of *faeD* expression, and it is responsible for *faeD* induction in the presence of ferulic acid.

Effect of ferulic acid on induction of the pectate lyase genes. During analysis of *faeD* expression, we also tested the effect of the potential inducers on pectate lyase production. We observed that ferulic acid weakly induced pectate lyase activity (Table 3). In the presence of polygalacturonate, the effect of ferulic acid was even clearer, and induction reached a 5-fold factor (Table 3). Similar effects on the expression of a transcriptional fusion with *pelD*, which encodes one of the major pectate lyases, were observed (Table 3). Thus, induction by ferulic acid takes place at the transcriptional level, and ferulic acid acts in synergy with polygalacturonate to increase the induction of pectate lyase genes. Plant extracts were previously shown to provoke an increased pectate lyase induction in the presence of polygalacturonate, and this phenomenon was

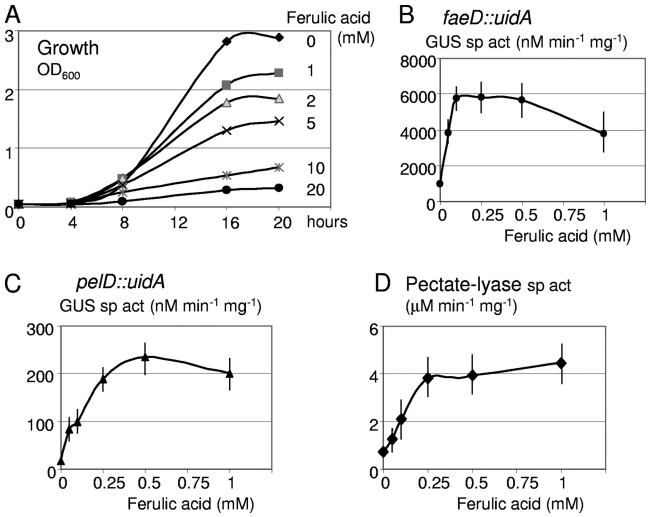

FIG. 7. Effect of ferulic acid on growth, *faeD* transcription, and pectate lyase production. (A) Growth of the wild-type *D. dadantii* strain was monitored in M63 medium supplemented with glycerol and different concentrations of ferulic acid. OD_{600}, optical density at 600 nm. (B to D) The β-glucuronidase (GUS) specific activity, reflecting the transcription of the *faeD*::*uidA* (B) or of the *pelD*::*uidA* (C) fusion, were determined at different concentrations of ferulic acid, as was the total pectate lyase specific activity (D).

TABLE 3. Effect of ferulic acid on the production of pectate lyases

Potential inducer(s)[a]	Sp act[b]	
	Pectate lyase	β-Glucuronidase (fusion *pelD*::*uidA*)
None	0.04	4
Ferulic acid	0.09	10
Polygalacturonate	1.04	29
Polygalacturonate + ferulic acid	5.01	235
Plant extract	0.24	
Polygalacturonate + plant extract	4.62	
Plant extract + ferulic acid	1.38	
Polygalacturonate + ferulic acid + plant extract	11.31	

[a] The potential inducers were added at the following concentrations: plant extract, 1%; polygalacturonate, 2 g liter^{-1}; ferulic acid, 0.5 mM.

[b] The pectate lyase specific activity is expressed in μmol min^{-1} mg^{-1}. The β-glucuronidase specific activity, reflecting the expression of the *pelD* transcriptional fusion, is expressed in nmol min^{-1} mg^{-1}. These results are the average values from at least 3 independent experiments, with standard deviations corresponding to less than 20%.

called hyperinduction, but the inducers were not identified (3, 14, 25). The presence of ferulic acid results in the same type of hyperinduction. However, the effects of ferulic acid and plant extract remained additive (Table 3), suggesting that ferulic acid is not the sole component of the plant extract with pectate lyase inducing activity. The effect of ferulic acid concentrations, ranging from 0.05 to 1 mM, was tested on the induction of the *pelD* fusion and on pectate lyase production (Fig. 7). The induction was maximal at ferulic acid concentrations of 0.25 to 0.5 mM. Thus, pectate lyase induction required a 5-fold-higher concentration of ferulic acid than *faeD* induction (Fig. 7).

Analysis of pectate lyase production in mutants with inactivation of the regulator KdgR, PecS, PecT, Pir, Crp, or GacA indicated that none of them are necessary for the induction of pectate lyase genes by ferulic acid (data not shown). Similarly, analysis of the *faeR* mutant indicated that, in contrast to *faeD* regulation, FaeR is not involved in the induction of pectate lyases in the presence of ferulic acid (data not shown).

Conclusion. The plant-pathogenic bacterium *D. dadantii* produces a large array of plant cell wall-degrading enzymes, including several pectinases. Using an *in situ* detection test, we showed that it also produces two feruloyl esterases, FaeD and FaeT. These enzymes have been previously analyzed in a variety of microorganisms, and they have potential biotechnological and medicinal applications (2). Carbohydrate esterases are classified in different families on the basis of their amino acid sequences (http://www.cazy.org/), and the previously characterized feruloyl esterases are members of the carbohydrate esterase family CE1. While recent selections were usually based on the search for CE1 homologues, we used a functional screening to isolate new enzymes. This led us to identify two esterases of the family CE10 (Fig. 3), which also includes the *D. dadantii* pectin acetylesterase PaeX (31) and several noncarbohydrate active enzymes. Thus, FaeD and FaeT are the first two members of a new family of feruloyl esterases. It will be interesting to study their properties in detail and to clarify their specificity on natural substrates for comparison with CE1 enzymes. We have already seen strong activation of the FaeD and FaeT esterases by ferulic acid (Fig. 5B), which was not observed for CE1 feruloyl esterases. Indeed, feruloyl esterases of the FaeD-FaeT family may have particular properties that could be of interest in extending the biotechnological applications of these enzymes.

Inactivation of the *faeD* and *faeT* genes indicated major activity of FaeD in *D. dadantii* (Fig. 1). In a previous analysis of the *D. dadantii* secretome, FaeD was identified among the extracellular proteins secreted by the Out system, which mediates the secretion of most pectinases (18). In the present report, we have shown that *faeD* expression is specifically induced by ferulic acid. Such induction is mediated by FaeR, a regulator of the LysR family encoded by the divergent *faeD* gene. The *faeD-faeR* locus is conserved in all the sequenced genomes of pectinolytic enterobacteria of the genera *Dickeya* and *Pectobacterium*. The extracellular location of FaeD allows it to act on feruloylated polysaccharides. This location explains why the small product, ferulic acid, rather than the large substrate acts as an inducer of the *D. dadantii faeD* gene. The basal level of *faeD* expression may lead to the release of ferulic acid from plant polysaccharides, which would then activate *faeD* transcription at low concentrations (Fig. 7B). Increased feruloyl esterase activity gives rise to higher ferulic acid concentrations, which in turn, induce the production of pectate lyases (Fig. 7D) that cause the maceration symptom.

The induction of feruloyl esterases by phenolic compounds has been previously observed. The gene *cinB* of *Butyrivibrio fibrosolvens* is specifically induced by feruloylated oligosaccharides which are also CinB substrates (7). In the fungus *Aspergillus niger*, feruloyl esterase production is stimulated by the presence of the product, free ferulic acid (10). In *Aspergillus kawachii*, ferulic acid increased the production of feruloyl esterases and also that of various plant cell wall-degrading enzymes (19).

In *D. dadantii*, ferulic acid induces pectate lyase production by an unknown mechanism, independent of FaeR. For this induction, ferulic acid acts in synergy with the pectate lyase substrate polygalacturonate (Table 3), which when cleaved, liberates strong inducers (13). Such synergy has been previously observed with crude plant extracts (3, 25), and it is probably due to the effect of several plant compounds, including ferulic acid. It was recently shown that some phenolic compounds affect the expression of the type III secretion system of *D. dadantii*. While cinnamic and *o*-coumaric acids induce this system (39), *p*-coumaric acid represses its expression (23). The GacAS system was thought to be responsible for this induction (39). In contrast, GacA is not involved in the induction of pectate lyases by ferulic acid. These data suggest the involvement of a number of phenolic signals and response regulators in *D. dadantii*. Plant phenolic compounds may play an important role as signals in the plant-*D. dadantii* interaction.

Phenolic acids constitute an important class of organic compounds produced by plants, and several are recognized as signaling molecules by plant-associated microorganisms. The role of acetosyringone in the induction of *vir* genes of agrobacteria has been widely documented, somewhat masking the involvement of other phenolic compounds. The *Agrobacterium* tumefaciens strain KU12, which has a large host range, is induced by the common phenolic compounds ferulic and *p*-coumaric acids (22). Some simple phenolics, including ferulic acid, were also identified as chemoattractants and inducers of the *nod* genes in certain rhizobial species (17). The variety of

phenolic compounds, with differences in structures, amounts, and prevalence, may generate subtleties in the dialogues that take place during the plant-bacteria interactions.

Despite the fact that inactivation of the *faeD* or *faeT* gene did not reduce *D. dadantii* virulence, *faeD* is clearly expressed in the macerated tissue (Fig. 6C). Feruloyl esterases could have multiple influences on *D. dadantii* pathogenesis. The dissociation of internal cross-links in the polysaccharide network of the plant cell wall by feruloyl esterases could facilitate the access of the main-chain-degrading enzymes to the polysaccharide backbone. Suppression of the polysaccharide esterifications by feruloyl esterases could also favor the action of depolymerases. Finally, the liberation of ferulic acid by feruloyl esterases would contribute to the induction of pectate lyases, the main determinant of plant maceration. These different effects of feruloyl esterases facilitate soft rot disease caused by pectinolytic bacteria.

ACKNOWLEDGMENTS

Appreciation is expressed to Guy Condemine, Vladimir Shevchik, and Valerie James for reading the manuscript. We thank the other members of the Lyon *Erwinia* group for helpful discussions and the members of the International *Erwinia* Consortium for the exchange of unpublished data concerning the *D. dadantii* 3937 genome.

This work was supported by a Ph.D. grant from the Syrian government to S.H. and by grants from the Centre National de la Recherche Scientifique and from the Ministère de l'Enseignement Supérieur et de la Recherche.

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