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An Isoflavonoid-Inducible Efflux Pump in *Agrobacterium tumefaciens* Is Involved in Competitive Colonization of Roots

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Agrobacterium tumefaciens **1D1609, which was originally isolated from alfalfa (*Medicago sativa* L.), contains genes that increase competitive root colonization on that plant by reducing the accumulation of alfalfa isoflavonoids in the bacterial cells. Mutant strain I-1 was isolated by its isoflavonoid-inducible neomycin resistance following mutagenesis with the transposable promoter probe Tn*5*-B30. Nucleotide sequence analysis showed the transposon had inserted in the first open reading frame, *ifeA*, of a three-gene locus (*ifeA*, *ifeB*, and *ifeR*), which shows high homology to bacterial efflux pump operons. Assays on alfalfa showed that mutant strain I-1 colonized roots normally in single-strain tests but was impaired significantly ($P \leq 0.01$) in competition against wild-type strain 1D1609. Site-directed mutagenesis experiments, which produced strains I-4 (*ifeA*::*gusA*) and I-6 (*ifeA*::Ω-Tc), confirmed the importance of *ifeA* for competitive root colonization. Exposure to the isoflavonoid coumestrol increased β-glucuronidase activity in strain I-4 21-fold during the period when coumestrol accumulation in wild-type cells declined. In the same test, coumestrol accumulation in mutant strain I-6 did not decline. Expression of the *ifeA-gusA* reporter was also induced by the alfalfa root isoflavonoids formononetin and medicarpin but not by two triterpenoids present in alfalfa. These results show that an efflux pump can confer measurable ecological benefits on *A. tumefaciens* in an environment where the inducing molecules are known to be present.**

Agrobacterium tumefaciens causes crown gall tumors on a wide range of dicotyledonous plants by colonizing wounded tissues and transferring oncogenes into the plant genome (15). Successful interaction between *A. tumefaciens* and target cells of the host plant depends on the capacity of the bacteria to elude deleterious plant defense compounds that can slow growth. In the rhizosphere, *A. tumefaciens* also must compete effectively with other microorganisms as it colonizes the root (38).

Alfalfa roots release a wide variety of molecules, including many flavonoids (29). Isoflavonoids, a subgroup of flavonoids, have been studied both for their negative effects on microorganisms and for their role as inducers of nodulation genes in symbiotic *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium* spp. (8). Alfalfa roots are specifically known to store glucosides of the isoflavonoids formononetin, coumestrol, and medicarpin (42), as well as saponins of the hydrophobic triterpenoids hederagenin and medicagenic acid (21). Root exudates from this species have been shown to contain various forms of coumestrol, formononetin, and medicarpin (7, 19). One can therefore ask whether bacteria which colonize alfalfa roots have evolved any particular mechanism either to use or to avoid these compounds.

The first *A. tumefaciens* naturally infective on alfalfa was isolated recently from a crown gall. This strain, designated 1D1609, is exceptionally virulent on alfalfa (27). Studies with strain 1D1609 showed that virulence on alfalfa depended not only on the Ti plasmid but also on undefined chromosomal loci that were absent in other *A. tumefaciens* strains. These loci could play many roles, but it is reasonable to postulate their involvement with factors known to be present in the alfalfa rhizosphere. We hypothesized these loci might confer some beneficial interaction with isoflavonoids exuded from alfalfa roots. To test this hypothesis, a mutant bank of *A. tumefaciens* 1D1609 was generated with the transposable promoter probe Tn*5*-B30, which carries a promoterless *nptII* gene (40). Screening for flavonoid- and isoflavonoid-inducible neomycin resistance found one particularly responsive locus which is identified here as an isoflavonoid efflux pump involved in the competitive colonization of alfalfa roots.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. Strains and plasmids used in this study are listed in Table 1. *A. tumefaciens* strains were grown in AB mineral medium (5) or LB medium (37) at 30°C with shaking (200 rpm). D1M agar, a selective medium for *A. tumefaciens*, contained (per liter) 5 g of cellobiose, 3 g of K_2HPO_4, 1 g of NaH_2PO_4, 1 g of NH_4Cl, 0.3 g of $MgSO_4 \cdot 7H_2O$, 10 mg of malachite green, and 15 g of agar. *Escherichia coli* strains were grown in LB medium. When appropriate, media were supplemented with tetracycline (2 μg/ml for *A. tumefaciens* and 10 μg/ml for *E. coli*), neomycin (80 μg/ml), ampicillin (50 μg/ml), or gentamicin (25 μg/ml). Standard chemicals, reagents, and antibiotics were purchased from Fisher Scientific (Santa Clara, Calif.) or Sigma Chemical Co. (St. Louis, Mo.).

Unless noted otherwise, flavonoids were purchased from Spectrum Chemical MFG Corp. (Gardena, Calif.). Coumestrol was purchased from Eastman Kodak Co. (Rochester, N.Y.). The 4,4′-dihydroxy-2′-methoxychalcone was synthesized (6). Medicarpin and the triterpenoids hederagenin and medicagenic acid were isolated as glycosides from mature alfalfa roots, hydrolyzed to aglycones, and purified by high-pressure liquid chromatography (21, 22, 26, 36). All compounds obtained by purification or synthesis were confirmed by nuclear magnetic resonance and mass spectrometry against published values. Flavonoids, isoflavonoids, and triterpenoids were prepared as 2.5 mM stock solutions in 100% methanol.

Transposon mutagenesis and screening. Mutants were constructed by conjugating pSUP102::Tn*5*-B30 from *E. coli* S17-1 into *A. tumefaciens* 1D1609 under standard conditions (39). Transposon mutants were selected on D1M agar containing 2 μg of tetracycline per ml and maintained in microtiter plates in AB medium with the same antibiotic. Inducibility of the *nptII* promoter probe from Tn*5*-B30 was screened in mutants by testing for differential neomycin resistance in the presence or absence of isoflavonoids and flavonoids. Mutants were repli-

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TABLE 1. Strains, plasmids, and transposons

Strain, plasmid, or transposon	Characteristics	Source or reference
A. tumefaciens		
1D1609	Wild-type alfalfa isolate, Km^r Sp^r	27
I-1	*ifeA*::Tn*5*-B30 mutant of 1D1609	This study
I-4	*ifeA*::*gusA* mutant of 1D1609	This study
I-6	*ifeA*::Ω-Tc mutant of 1D1609	This study
E. coli		
DH5α	*recA1* Δ*lacZ*	Gibco BRL
HB101	pRK2013 helper strain for matings	Stratagene
S17-1	Modified RP4 integrated into genome	39
VCS257	Host strain for cosmid libraries	Stratagene
Plasmids and transposons		
pUC19	Multicopy cloning vector	Gibco BRL
pBSK+	Multicopy cloning vector	Stratagene
pJQ200mp18	pACYC184*ori*, *mob sacB* Gm^r	33
pRK2013	pRK212.2 derivative for matings	11
pSUP205	pBR325 derivative, *mob* λ*cos* Cm^r Tc^r	39
Tn*5*-B30	pSUP102 with transposable *nptII* promoter probe, Tc^r	40
Ω-Tc	pUT::mini-Tn*5Tc*, Tc^r	9
pCAM140	mini-Tn*5*SS*gusA40*, promoterless *gusA*	44
pAC107	pSUP205 with 1D1609 *ifeABR* locus	This study
pMC11	pSUP205 with I-1 *ifeA*::Tn*5*-B30 locus	This study
pMC115	pUC19 with *Sal*I fragment upstream of Tn*5*-B30 from pMC11	This study
pMC113	pUC19 with *Sal*I fragment downstream of Tn*5*-B30 from pMC11	This study
$pAC1073^+$	pBSK+ with 5.2-kb *Eco*RI *ifeABR′* fragment of pAC107	This study
$pAC1073^-$	pBSK+ with 5.2-kb *Eco*RI *ifeABR′* fragment from pAC107	This study
pAC1074	pJQ200mp18 with 0.9-kb *Eco*RI-*Sal*I fragment of pAC1073+	This study
pAC1075tet	pAC1074::Ω-Tc	This study
pAC1075gus	pAC1074::*gusA*	This study
pAC1079	pUC19 with 7.5-kb *Pst*I *ifeABR* fragment of pAC107	This study
p18Not	*Not*I-*Eco*RI-*Sal*I-*Hin*dIII-*Not*I as MCS in pUC18	13

cated from microtiter plates onto AB agar containing 2 μg of tetracycline per ml, 80 μg of neomycin per ml, and either 0.2% methanol (negative control)–1 μM coumestrol or a mixture of 10 μM each of formononetin, quercetin, luteolin, 4′,7-dihydroxyflavone, and 4,4′-dihydroxy-2′-methoxychalcone. Mutants which grew better on medium containing the flavonoid-isoflavonoid mixture or coumestrol than on medium containing methanol were retested for induced neomycin resistance on agar-containing individual compounds.

Rhizosphere tests. Assays for bacterial colonization of alfalfa (*Medicago sativa* L. cv. CUF101) roots were performed in vermiculite as described previously (41) with the following modifications. Plant nutrient solution was supplemented with 8 mM NH_4NO_3. Inocula were prepared by growing cultures of strains 1D1609, I-1, and I-6 overnight in AB medium, washing once in sterile dilution buffer (25 mM NaH_2PO_4, 25 mM Na_2HPO_4, 0.01% Tween 20; pH 7.0), and resuspension in dilution buffer to an optical density of 0.5. In single-strain inoculations, suspensions of each strain were diluted to 1×10^3 to 3×10^3 CFU/ml; for dual-strain inoculations, suspensions of strain 1D1609 and either strain I-1 or strain I-6 were mixed 1:1 and diluted to 1×10^3 to 3×10^3 CFU/ml. At each sampling point, bacteria were recovered as described previously (41) from entire plant roots of uniform size. Serial dilutions of bacterial suspensions were plated on AB agar with and without tetracycline (2 μg/ml) for enumeration. Data for each sampling point consisted of bacterial counts recovered from 7 to 10 plants. Each root colonization experiment was repeated at least three times.

Molecular analysis of mutant strain I-1. Total DNA from strain I-1 was isolated from cells grown overnight in LB medium and collected by centrifugation (3 min at 10,000 × *g*). Cell pellets were resuspended in 200 μl of TEN buffer (10 mM Tris, 1 mM EDTA, 10 mM NaCl; pH 8.0). Cells were lysed by adding 100 μl of proteinase K solution (1 mg/ml in TEN buffer) and 100 μl of sodium dodecyl sulfate (5% in TEN buffer) and incubating 1 h at 37°C. NaCl was then added to a final concentration of 0.5 M, and lysates were incubated at 68°C for 30 min before extracting twice with buffer-saturated phenol-chloroform and twice with chloroform. DNA was precipitated with an equal volume of cold isopropanol and washed twice with 70% ethanol. Precipitated DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). Restriction digests (37) were performed with commercial enzymes (Promega, Madison, Wis.).

Cosmid clones of strain I-1 DNA were prepared by standard protocols (14), and clones containing the DNA fragment with the Tn*5*-B30 insertion were isolated by selecting for tetracycline resistance conferred by Tn*5*-B30. Clone pMC11 was selected on LB agar with tetracycline, and a physical map was constructed from restriction digests and hybridization tests with Tn*5*-B30 DNA which was labeled randomly with a Genius digoxigenin kit (Boehringer, Mannheim, Germany). Hybridizations were performed at 68°C and washed under high-stringency conditions according to the manufacturer's instructions. *Sal*I fragments of pMC11 corresponding to chromosomal DNA flanking Tn*5*-B30 were subcloned into *Sal*I-digested pUC19 to construct pMC115 and pMC113 containing the *Sal*I fragments upstream and downstream of the Tn*5*-B30 insertion in pMC11, respectively. A DNA probe specific to the mutated locus in I-1 was prepared by PCR amplification of the subcloned *Sal*I fragment in pMC113 with primers *Tn5out* (5′ GAA AGG TTC CGT TCA GGA CGC TAC 3′) and *M13R* (5′ TCA CAC AGG AAA CAG CTA TGA C 3′), followed by random labeling of this product with digoxigenin.

A genomic cosmid library was prepared in pSUP205 by using wild-type DNA from strain 1D1609, and clone pAC107 containing DNA corresponding to the locus mutated in strain I-1 was identified by colony hybridization by using the probe prepared from pMC113. The 5.2-kb *Eco*RI fragment from clone pAC107 corresponding to the mutated locus in strain I-1 was subcloned into pBSK+ in both orientations to create $pAC1073^+$ and $pAC1073^-$. Nested deletions for sequencing were prepared from *Xba*I- and *Sac*I-digested $pAC1073^+$ and $pAC1073^-$ DNA with a double-stranded nested deletion kit (Pharmacia Biotech, Piscataway, N.J.). Both DNA strands were sequenced automatically (ABI 377; Applied Biosystems Perkin-Elmer, Foster City, Calif.) with standard M13 primers at the Division of Biological Sciences DNA Sequencing Facility (University of California, Davis, Calif.). Assembled sequences were analyzed for similarities to known sequences by using the BLAST (1) internet site (http://www.ncbi.nlm.nih.gov/BLAST).

Site-directed mutations of *ifeA*. Plasmids for gene insertion within *ifeA* in strain 1D1609 were constructed in the *sacB* positive-selection suicide vector pJQ200mp18 (33). A 0.9-kb *Eco*RI-*Sal*I fragment of $pAC1073^+$ containing the first 639 bp of the *ifeA* open reading frame was subcloned into pJQ200mp18 to form pAC1074. A 2.0-kb *Eco*RI-generated DNA fragment containing the tetracycline resistance interposon Ω-Tc (10) was excised from mini-Tn*5Tc* (9) and cloned into p18Not (13). The resulting Ω-Tc *Not*I fragment was cloned into the unique *Not*I site within the *ifeA* fragment in pAC1074 to form pAC1075tet. A 2.0-kb *Not*I fragment containing a promoterless *gusA* gene was excised from pCAM140 (44) and cloned into the unique *Not*I site within the *ifeA* fragment in pAC1074 to form pAC1075gus. Plasmids pAC1075tet and pAC1075gus were

conjugated into *A. tumefaciens* 1D1609 by triparental mating by using pRK2013 (11).

Single-recombinant clones resulting from chromosomal integration of pAC1075tet were selected on AB agar containing tetracycline and gentamicin. Double-recombinant clones resulting from a second homologous recombination event, which deleted the vector portion of the introduced plasmid along with the wild-type *ifeA* fragment, were selected on AB agar containing tetracycline and 5% sucrose. Likewise, single-recombinant clones containing chromosomally integrated pAC1074gus were selected on AB agar containing gentamicin. Double-recombinant clones containing only *ifeA*::*gusA* were selected on AB agar containing 5% sucrose and screened for coumestrol-inducible β-glucuronidase (GUS) activity on AB medium containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (25 μg/ml).

Insertion of the *ifeA*::*gusA* gene fusion in mutant strain I-4 was confirmed by PCR by using primers *gusu* (5′ AGA CTG AAT GCC CAC AGG CCG TCG 3′) and *uncd* (5′ CAT GTC GTC CAT CCA TGT AGA TAG 3′) to amplify the fragment upstream of the insertion and primers *gusd* (5′ GCG TTG GCG GTA ACA AGA AAG G 3′) and *dclu* (5′ CTT CAC ACG ATC CAG ACG GAG 3′) to amplify the downstream fragment. Insertion of Ω-Tc within *ifeA* in mutant strain I-6 was confirmed by PCR by using primers Ω*out* (5′ CCG GTG GAT GAC CTT TTG AAT GA 3′) and *uncd* to amplify the upstream fragment and primers Ω*out* and *dclu* to amplify the downstream fragment.

Isoflavonoid accumulation tests. Coumestrol was added to a final concentration of 50 μM to cultures of wild-type strain 1D1609 and mutant strain I-6 grown to late exponential phase (optical density at 600 nm = 0.8 to 1.0). Triplicate samples (1 ml) of each culture were removed at selected time points. Cells were pelleted by centrifugation (3 min at 10,000 × *g*) and resuspended in methanol (1 ml) for 4 h at room temperature with shaking to extract coumestrol. The extraction was terminated by removing cells (3 min at 10,000 × *g*), and coumestrol in the supernatant was quantified by measuring the A_{342} value in a Lambda 6 dual-beam spectrophotometer (Perkin-Elmer, Norwalk, Conn.) relative to a standard curve (A_{342} versus concentration). The coumestrol content of culture samples was normalized to cell number and expressed as nmol/10^9 CFU. Each experiment was repeated twice.

Induction of *ifeA*::*gusA* expression. Coumestrol induction of *ifeA* expression was measured as GUS activity in cultures of strain I-4 which were treated identically and run in parallel to wild-type 1D1609 and mutant I-6 cells during the isoflavonoid accumulation tests. After cells were centrifuged (3 min at 10,000 × *g*) out of the coumestrol-containing medium, pellets were washed once in carbon substrate-free AB medium containing 100 μg of chloramphenicol per ml and resuspended in 1 ml of the same medium. GUS assays measured the hydrolysis of *p*-nitrophenylglucuronide (43), and GUS activity was normalized to cell number as nanomoles of *p*-nitrophenylglucuronide hydrolyzed/min/10^9 CFU. Each experiment was repeated twice.

Induction of *ifeA* was assayed as GUS activity in cultures of strain I-4 which were grown overnight in the presence of the test compound. Exponentially growing cultures of strain I-4 in AB medium were diluted 100-fold into AB medium containing different concentrations of either coumestrol, formononetin, medicarpin, genistein, daidzein, biochanin-A, quercetin, 4,4′-dihydroxy-2′-methoxychalcone, hederagenin, or medicagenic acid and were grown overnight. GUS assays were performed and quantified as described above, with triplicate samples (1 ml) of each treatment. Each treatment was repeated twice.

RESULTS

Isolation of flavonoid-inducible mutants. Approximately 5,000 Tn*5*-B30 mutants of strain 1D1609 were screened for inducible neomycin resistance in the presence of a mixture of flavonoids and isoflavonoids. These tests identified mutant strain I-1 as showing a reproducible induction of neomycin resistance, and further experiments with individual flavonoid and isoflavonoid compounds indicated that the promoterless *nptII* insertion in strain I-1 was induced by 1 μM coumestrol and 10 μM formononetin (Fig. 1).

Rhizosphere competence of mutant strain I-1. Wild-type strain 1D1609 and mutant strain I-1 achieved identical colonization densities on alfalfa roots 10 days after they were inoculated in single-strain tests (Fig. 2A), but the mutant competed poorly with wild-type cells when a 1:1 mixture of the two strains was introduced (Fig. 2B). On day 10 in the competitive assay, the ratio of mutant I-1 to wild-type 1D1609 cells was 0.33. Both strains grew rapidly in the first 2 days after inoculation when cell densities were low, and no effect of the mutation was detected at that time. These genotypic effects on alfalfa root colonization were seen in three independent experiments.

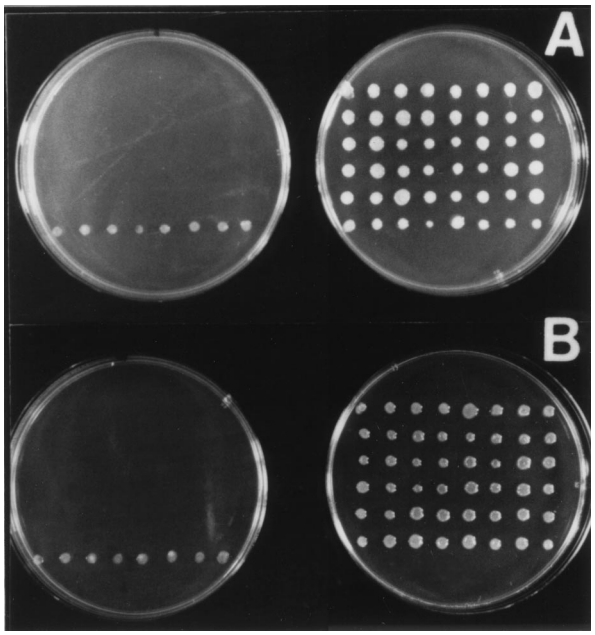

FIG. 1. Identification of an *A. tumefaciens* strain mutated in an isoflavonoid-inducible locus by the promoterless reporter gene *nptII* which confers neomycin resistance. (A) Growth of mutant strain I-1 on neomycin-containing medium with (right) or without (left) 10 μM formononetin. (B) Growth of mutant strain I-1 on neomycin-containing medium with (right) or without (left) 1 μM coumestrol. AB medium was supplemented with 0.2% methanol as a control (left) for the isoflavonoid solutions (right). All plates were inoculated with 40 replicate colonies of mutant strain I-1 (top five rows) and eight replicate colonies of a constitutively neomycin-resistant mutant (bottom rows).

Molecular analysis of mutant strain I-1. Hybridization tests of total DNA from strain I-1 with a Tn*5*-B30-specific DNA probe indicated that strain I-1 contained a single transposon insertion within a 5.2-kb *Eco*RI chromosomal restriction fragment. Colony hybridization tests of a wild-type 1D1609 DNA library with a probe specific for the flanking region downstream of the Tn*5* insertion in mutant I-1 located four overlapping cosmid clones. These clones contained the same restriction pattern as that of the mutated locus in strain I-1 (Fig. 3). The 5.2-kb *Eco*RI fragment in pAC107 was sequenced and revealed a 5,227-bp fragment with two complete open reading frames and part of a third. The sequence of this third open reading frame was extended and completed by using sequence-specific primers and template DNA from pAC1079, which contains the *Pst*I restriction fragment from pAC107 (Fig. 3). Sequence data for all three open reading frames were submitted to the GenBank (National Center for Biotechnology Information [NCBI]) database as accession number AF039653.

Sequence similarity searches with the NCBI BLAST database tool indicated that the predicted proteins encoded by these open reading frames are highly similar to proteins encoded by efflux system genes in other bacteria (Table 2). The first open reading frame, *ifeA* for *i*so*f*lavonoid *e*fflux, encodes a predicted protein of 384 amino acids with amino acid similarity (23 to 34% identical residues and 37 to 50% conserved residues) to members of a family of membrane-fusion proteins. The second open reading frame, *ifeB*, encodes a predicted protein of 1,046 amino acids with amino acid similarity (37 to 49% identical residues and 54 to 65% conserved residues) to members of a family of transmembrane efflux pump proteins. The third open reading frame, *ifeR*, encodes a predicted protein of 208 amino acids with amino acid similarity (27 to 49%

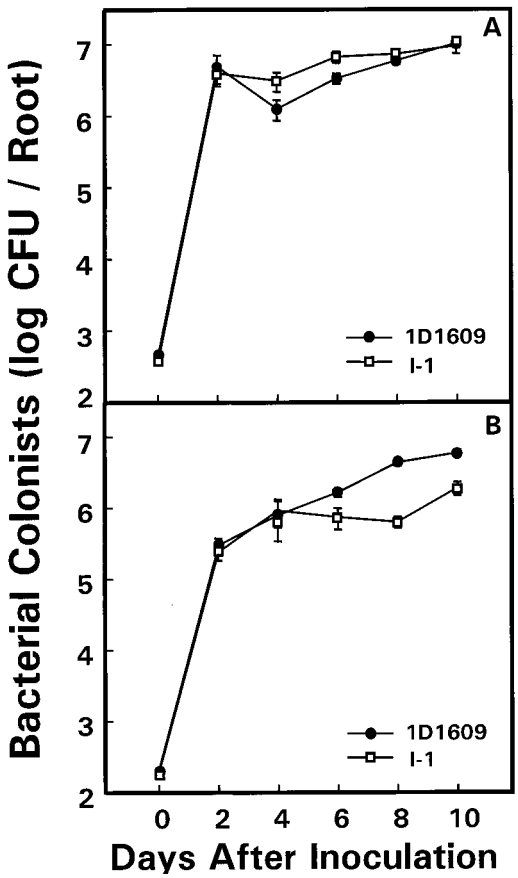

FIG. 2. Alfalfa root colonization by wild-type *A. tumefaciens* 1D1609 and mutant strain I-1. Strains were inoculated separately (A) or as a 1:1 mixture (B) on sterile alfalfa seedlings at the time of germination. Root-colonizing bacteria were recovered and counted by dilution plating at the times indicated. Bacterial counts are reported as means ± standard errors from 7 to 10 replicate plants. Standard error bars are obscured by symbols in some cases.

TABLE 2. Similarity of *ifeA*, *ifeB*, and *ifeR* open reading frames to efflux pump proteins in other bacteria

Putative homologous protein (no. of amino acids)	% Identical residues	% Conserved residues	Reference
IfeA (384)			
E. coli			
AcrA (397)	33	49	20
AcrE (385)	34	48	16
EnvC (384)	31	46	17
P. aeruginosa			
MexA (383)	30	41	32
MexC (387)	29	43	31
MexE (414)	23	37	18
N. gonorrhoeae MtrC (412)	34	50	28
IfeB (1,046)			
E. coli			
AcrB (1,049)	49	65	20
AcrF (1,034)	48	63	16
AcrD (1,034)	46	60	4
P. aeruginosa			
MexB (1,046)	48	64	32
MexD (1,043)	40	55	31
MexF (1,062)	37	54	18
N. gonorrhoeae MtrD (1,067)	43	62	12
IfeR (208)			
E. coli			
AcrR (215)	32[a]	52[a]	20
EnvR (220)	27[a]	53[a]	16
N. gonorrhoeae MtrR (210)	49[b]	63[b]	28

[a] Sequence similarity to N-terminal 113 amino acids.
[b] Sequence similarity to N-terminal 61 amino acids.

identical residues and 52 to 63% conserved residues) to regulatory proteins of homologous efflux pump operons over the N-terminal regions. Sequence analysis of chromosomal DNA flanking the Tn*5*-B30 insertion in strain I-1 showed that the transposon inserted 320 bp downstream from the start codon for *ifeA*. Characteristic of Tn*5* transposition, the insertion of Tn*5*-B30 in strain I-1 resulted in the duplication of 9 bp (5′ GGCCAATGT 3′) flanking the site of transposition.

Verification of the *ifeA* mutant phenotype. Mutant strain I-6 was constructed to contain an insertion of Ω-Tc, creating a polar mutation within *ifeA*. Successful construction was confirmed by PCR amplification of fragments flanking the site of Ω-Tc insertion and by DNA hybridization of total I-6 DNA to the pAC1074-derived *ifeA* probe, indicating an insertion of ~2.0 kb within *ifeA* (data not shown).

Competitive root colonization tests established that the insertion in *ifeA* in strain I-6 impaired competitiveness against strain 1D1609 in a manner similar to the insertion in *ifeA* in strain I-1 (Table 3). In three separate experiments, strain I-6 was significantly ($P \leq 0.01$) less competitive than wild-type 1D1609 when measured in populations recovered from alfalfa roots 10 days after inoculation. The absolute values measured for competition in strains I-1 and I-6 (i.e., the recovery ratio of mutant to wild type) of 0.33 and 0.27 were not significantly different. These data indicate that the insertion of Ω-Tc in *ifeA* in strain I-6 was phenotypically equivalent to the insertion of Tn*5*-B30 in *ifeA* in strain I-1.

Isoflavonoid accumulation in *A. tumefaciens* cells. Within 5 min after its addition, coumestrol accumulated to a significantly ($P \leq 0.01$) higher level in mutant strain I-6 than in the wild-type cells (Fig. 4). Coumestrol levels in mutant cells remained nearly twice as high as in wild-type cells for nearly 1 h, and then the difference increased markedly as the coumestrol

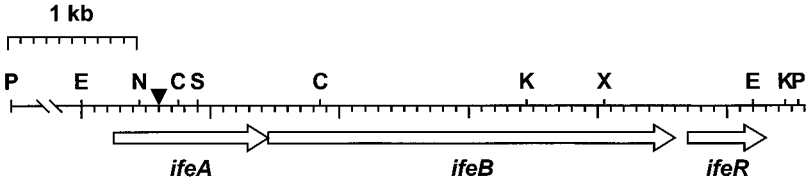

FIG. 3. Restriction map of the 7.5-kb *Pst*I fragment containing the Tn*5*-B30 insertion (▼) in *A. tumefaciens* mutant strain I-1. Restriction sites are represented as C, *Cla*I; E, *Eco*RI; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sal*I; or X, *Xho*I. Arrows below the map indicate open reading frames predicted from nucleotide sequence analysis.

TABLE 3. Competitive alfalfa root colonization by wild-type and mutant I-6 (*ifeA*::Ω-Tc) cells of *A. tumefaciens* 1D1609

Expt	Coinoculated cells[a]	Recovered titer (10^6 CFU/plant)[b]	Recovered ratio (I-6/1D1609)[b]
1	I-6	1.28 ± 0.28	0.28 ± 0.08
	Wild type	5.19 ± 0.53	
2	I-6	1.10 ± 0.15	0.27 ± 0.05
	Wild type	4.74 ± 0.71	
3	I-6	2.30 ± 0.91	0.27 ± 0.09
	Wild type	8.19 ± 1.06	

[a] Cells were coinoculated at a 1:1 ratio on day 0 with a total of 1×10^2 to 3×10^2 CFU/plant.
[b] Mean titers and ratios ± standard error of bacteria recovered from 7 to 10 plants 10 days after inoculation.

content in wild-type cells decreased. At the end of the 120-min experiment, the coumestrol content of mutant cells was about 20-fold higher than that of the wild-type cells because coumestrol remained at high, unchanged levels in mutant strain I-6. Reduction of coumestrol in wild-type cells was associated with a 21-fold increase in expression of the *ifeA-gusA* reporter fusion in strain I-4 exposed to the same conditions (Fig. 4). We interpret these data as showing that an efflux pump associated with *ifeA* expression was responsible for the dramatic decline in the coumestrol content of wild-type cells during the second hour of this experiment. Similar results were obtained in two separate experiments.

Induction of *ifeA* in *A. tumefaciens*. Expression of the *ifeA*::*gusA* fusion in strain I-4 was induced by several flavonoids but not by two triterpenoids after overnight growth in media containing the test compounds (Table 4). GUS expression was significantly higher ($P \leq 0.05$) in strain I-4 cells grown in the presence of the alfalfa isoflavonoids coumestrol, medicarpin, and formononetin or the soybean isoflavonoids genistein, daidzein, and biochanin-A. The alfalfa chalcone 4,4′-dihydroxy-2′-methoxychalcone also strongly induced expression of *ifeA*, but

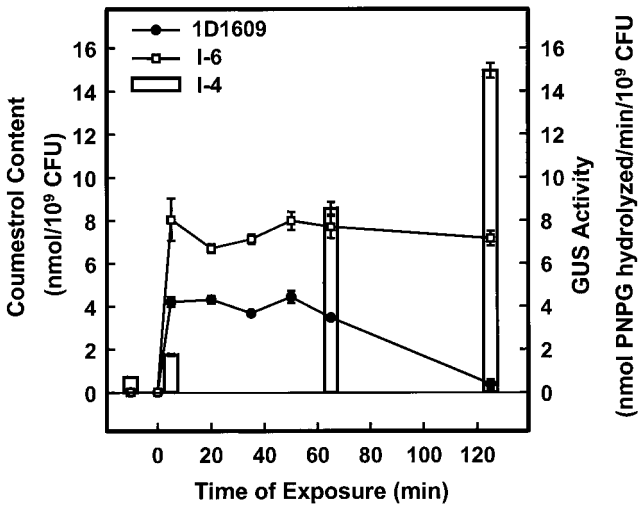

FIG. 4. Coumestrol accumulation and *ifeA* expression in *A. tumefaciens*. Coumestrol accumulation in wild-type strain 1D1609 (●) and mutant strain I-6 (□) was measured before and after the addition of 50 μM coumestrol (left axis). Corresponding expression of *ifeA* was measured as GUS activity (bar graph) in mutant strain I-4 (right axis). Data represent means ± standard errors from three replicates.

TABLE 4. Induction of *ifeA* expression by flavonoids and triterpenoids

Inducer	Inducer concn[a]	
	10 μM	50 μM
Coumestrol	9.8 ± 0.8	9.4 ± 1.6
Formononetin	1.7 ± 0.1	2.7 ± 0.2
Medicarpin	3.2 ± 0.2	17 ± 0.6
Genistein	4.8 ± 0.4	15 ± 1.6
Daidzein	4.1 ± 0.1	16 ± 1.8
Biochanin-A	1.4 ± 0.1[b]	4.0 ± 0.3
4,4′-Dihydroxy-2′-methoxychalcone	5.9 ± 0.8	9.9 ± 0.2
Quercetin	1.2 ± 0.1[b]	2.9 ± 1.0[b]
Hederagenin	1.0 ± 0.1[b]	1.4 ± 0.1[b]
Medicagenic acid	1.1 ± 0.1[b]	1.4 ± 0.2[b]

[a] Fold induced GUS activity after overnight induction, presented as the mean ± standard error relative to methanol control.
[b] Not significantly different from uninduced levels at $P \leq 0.05$.

the flavonol quercetin, which is released in large amounts by germinating seeds of many *Medicago* species, including alfalfa (30), gave a variable response which was not significant. These results indicate that at least six isoflavonoids and one chalcone can serve as natural inducers of *ifeA*.

DISCUSSION

Evidence provided here shows that a new genetic locus, *ifeABR*, contributes significantly to the ecological competence of *A. tumefaciens* 1D1609 in its normal habitat by reducing cellular accumulation of isoflavonoids. The *ifeA* locus is induced by various isoflavonoids and a chalcone (Fig. 1 and 4; Table 4), which are present in alfalfa root exudate (7, 19, 24). Both random (strain I-1) and site-directed (strain I-6) mutations in *ifeA* impaired competitive root colonization significantly (Fig. 2B; Table 3). As the first microbial pumping system described for isoflavonoids, these observations indicate that the well-established phenomenon of hydrophobic efflux pumps (3) confers ecological benefits in the rhizosphere ecosystem. Interestingly, this locus was absent from *A. tumefaciens* C58 and Ach5 and *Sinorhizobium meliloti* 1021 as determined by DNA hybridization to *ifeA* probes (data not shown).

Functional interpretation of *ifeABR* as an isoflavonoid efflux pump operon in *A. tumefaciens* is based on its capacity to reduce accumulation of coumestrol (Fig. 4). This result was consistent with an efflux pumping of coumestrol after it accumulated to a level which induced expression of *ifeA*. Coumestrol was used as a substrate in these experiments because it was identified as an inducer of *ifeA* expression (Fig. 1 and 4; Table 4). The polar mutation in *ifeA* in strain I-6 presumably disrupted expression of both *ifeA* and *ifeB* and resulted in the absence of an active efflux pump system. Additional work is required to define the physiological functioning of this putative isoflavonoid pump, including an investigation of the effect of proton gradient uncouplers on coumestrol accumulation, but the rhizosphere phenotype associated with its absence (Fig. 2B) establishes its ecological significance. Wild-type strain 1D1609 shows an unusual, strong resistance to kanamycin (27), but that trait was not affected by mutations in the *ifeA* gene.

Structural interpretation of the *ifeABR* locus as an efflux pump is based on sequence analysis relative to reported proteins. The open reading frames found in this study are predicted to code for proteins that are quite similar to known membrane-fusion proteins, transmembrane transporter proteins, and regulatory proteins (Table 2). These proteins appear

to belong to the "resistance-nodulation-division" family of efflux pump operons in gram-negative bacteria (25, 35). In cells expressing them, these operons confer resistance to a broad spectrum of hydrophobic and amphiphilic agents, including antibiotics, dyes, and nonionic detergents. The proposed mechanism of action of these pumps is that the transporter pump protein captures hydrophobic or amphiphilic substrate molecules from the cytoplasmic membrane and pumps them, via proton antiport, through a channel between the inner and outer membranes created by the membrane-fusion protein and possibly an outer-membrane porin channel (3, 25, 35). The generally hydrophobic nature of isoflavonoids makes them good candidates for such an efflux mechanism. However, since the growth of neither wild-type nor *ifeA* mutant cultures was inhibited by alfalfa isoflavonoids at concentrations of up to 50 μM (data not shown), *ifeABR* may be only one of several mechanisms conferring isoflavonoid resistance. This suggestion was supported by high-pressure liquid chromatography analyses, which showed that both mutant and wild-type cultures modified coumestrol (data not shown). It is also possible that the apparent *ife* efflux pump protects cells from rhizosphere compound(s) other than isoflavonoids.

Expression of efflux systems in other bacteria is regulated by signals of environmental stress (20, 32) and by efflux pump substrates (3). Induction of *ifeA* in *A. tumefaciens* by coumestrol (Fig. 4) may reflect the role of coumestrol as both an environmental signal and a pump substrate signal in the expression of the putative *ifeABR* efflux system. The broad specificity of known efflux pumping systems was mirrored to some extent by the tests for the specificity of *ifeA* expression (Table 4). In those experiments, the major isoflavonoids known to be present in alfalfa root exudate, as well as structurally related molecules such as genistein and daidzein, which are associated with soybean roots (8), were active inducers of *ifeA*. Whether the *ifeABR* locus confers any competitive advantage on *A. tumefaciens* 1D1609 in the rhizosphere of other legumes or nonlegume plants is not known.

Additional studies of the *ifeABR* locus are required to develop the depth of information already reported for many bacterial efflux pumps. For example, the transcriptional regulation of *ifeB* and the functional role of *ifeR* are not known. However, the identification here of an isoflavonoid-regulated locus which shows functional and structural similarities to known efflux pumps in bacteria offers a new direction for basic studies in rhizosphere ecology. The significant contribution of *ifeA* to competitive root colonization (Fig. 2B; Table 3) suggests that at least one rhizosphere bacterium reduces its exposure to isoflavonoids by a mechanism that differs from the widely recognized capacity of microorganisms to catabolize flavonoids (34) and isoflavonoids (2, 23).

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